BACK TO THE FUTURE: PACIFIC WALRUS STRESS RESPONSE AND REPRODUCTIVE STATUS IN A CHANGING ARCTIC

By

Patrick Maron Charapata, B.S.

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

in

Marine Biology

University of Alaska Fairbanks

August 2016

APPROVED:

Dr. Larissa Horstmann, Committee Co-Chair
Dr. Nicole Misarti, Committee Co-Chair
Dr. Matthew Wooller, Committee Member
Dr. Sarah M. Hardy, Program Head
Marine Sciences and Limnology Program
Dr. S. Bradley Moran, Dean,
School of Fisheries and Ocean Sciences
Dr. John Eichelberger
Dean of the Graduate School

Abstract

The Pacific walrus (*Odobenus rosmarus divergens*) is an iconic Arctic marine mammal that Alaska Natives rely on as a subsistence, economic, and cultural resource. A decrease in critical sea ice habitat and uncertain population numbers have led to walruses being listed as a candidate for the Endangered Species Act. However, there is no clear understanding of how walruses might be affected by climate change. The first objective of this study was to describe how bone steroid hormone concentrations relate to commonly used blubber and serum steroid hormone concentrations (i.e., cortisol, estradiol, progesterone and testosterone), because steroid hormones have not been extracted from marine mammal bone until now. Bone, blubber, and serum were collected from individual adult walruses (n = 34) harvested by Native Alaskan subsistence hunters during 2014 and 2015. Complete turnover of cortical bone in a walrus skeleton was estimated as ~33 years, approximately the lifetime of a walrus. Results showed bone and blubber steroid hormone concentrations were similar (P = 0.96, 0.51, 0.27 for cortisol, estradiol, and progesterone (males only), respectively), but not testosterone (males and females, P = 0.003) nor progesterone in blubber of female walruses (P = 0.007). Progesterone concentrations in males were significantly correlated between bone and blubber (R^2 = 0.51, P < 0.001). Estradiol measured in bone had high interannual variability (P < 0.001), indicating a shorter reservoir time in cortical bone compared with other hormones in this study, possibly due to local production of estradiol in walrus bone. Overall, bone serves as a long-term reservoir of steroid hormone concentrations compared with circulating serum concentrations. Progesterone measured in blubber can be compared with bone progesterone, but local production of estradiol in bone should be taken into account when interpreting these concentrations in cortical bone. The second objective of this study was to understand the physiological resiliency of walruses to the

current warming in the Arctic. Steroid hormone concentrations were measured in walrus bone collected from archaeological (n = 38, > 200 calendar years before present (BP)), historical (n = 135, 200 – 20 BP), and modern (n = 47, 2014 – 2015) time periods, but were also analyzed at a finer decadal (1830s – 2010s) scale. Walrus bone cortisol concentrations measured in modern-day walruses were similar to other time periods (P = 0.38, 0.07, for archaeological and historical time periods, respectively) indicating no increase in the stress response of walruses as a result of current sea ice conditions in the Arctic. Estradiol (females only), progesterone, and testosterone were significantly negatively correlated with walrus population estimates (P = 0.008, 0.003, < 0.001, respectively). A negative correlation indicates that walrus population numbers are low when reproductive hormone concentrations are high, and population numbers are high, possibly at carrying capacity, when hormone concentrations are low. Data from the current decade (2014 – 2015) show that the current walrus population has lower reproductive hormone concentrations compared to times of rapid population increase. These data indicate the present-day walrus population may not be increasing, but is either experiencing low calf production and / or is near its carrying capacity. Overall, these data provide walrus management with insights into the physiological resiliency of walruses in response to arctic warming, and validate bone as a valuable tissue for monitoring long-term physiological changes in the walrus population.

Dedication

This thesis is dedicated to my mom, Geralyn Charapata-Marsh. Thank you for your love and support of my ambitions to pursue a life in marine biology.

Table of Contents

List of Figures

Page

List of Tables

Page

List of Appendices

Page

Acknowledgments

This research was part of the Walrus Adaptability and Long-term Responses: Using multi-proxy data to project Sustainability (WALRUS) project funded through the National Science Foundation (NSF) with additional funding provided by Bureau of Ocean Energy Management (BOEM) awarded to PI and Co-Chair on my committee, Dr. Nicole Misarti (UAF), and Co-PI and Co-Chair on my committee, Dr. Lara Horstmann (UAF). This work was also supported in part by a UAF Center for Global Change Student Research Grant with funds from the Cooperative Institute for Alaska Research. Other UAF grants and scholarships helped support this research, including a Travel Grant from the UAF Graduate School and the Dr. Donald Hood Memorial Scholarship for Marine Science.

I would first and foremost like to thank my committee members, Drs. Lara Horstmann (Co-Chair), Nicole Misarti (Co-Chair), and Matthew Wooller (UAF). I am glad I "pestered" Dr. Lara Horstmann for months *via* email asking to be a part of her lab, because I ended up taking part in a unique project. Thank you for always making time for me to talk over data, presentations, and giving me a great graduate student experience. Thank you to Dr. Nicole Misarti for mentoring me as a graduate student, while knowing I had very little experience with bones and archaeology. I appreciate your expertise, help, and positive energy throughout this project, which has helped me greatly increase my knowledge in the zooarchaeology and general "bone" field. Dr. Matthew Wooller, thank you for your comments on my work in committee meetings and revisions on the various chapters throughout this project. Your positive attitude and interest in this project helped greatly throughout my time here at UAF. I will always be thankful to all three committee members, because they helped shape me into a better writer and research scientist throughout this research project.

This project would not be possible without the collaboration and support of Native Alaskan hunters from Gambell and Savoonga on St. Lawrence Island, as well as hunters from Barrow, Alaska. Thank-you to the Alaska Department of Fish and Game, Eskimo Walrus Commission, North Slope Borough, and U.S. Fish and Wildlife Service for your interest and continuing support of collecting samples for this project. Thank-you to the University of Alaska Museum, for special access to the Pacific walrus collection for sampling, which immensely helped build this dataset. Specifically, I would like to thank Dr. Link Olson, Aren Gunderson, Scott Shirar, Dr. Josh Ruther, and Kyndall Hildebrandt, for constantly opening up the museum's range doors for myself and others to sample walruses. Thank-you to the National Museum of Natural History for allowing sampling of the Pacific walrus collection and helping fill in data gaps. The majority of archaeological samples for this project came about through a huge effort by Dr. Anne Jensen. Thank you for coming to Fairbanks and spending time in the museum sorting through archaeological walrus and caribou bone for sampling. LC/MS/MS analyses of all our samples were done through a collaboration with Purdue University - Bindley Bioscience Center and Amber Jannasch. Thank you, Amber, for all the phone calls, emails, and visits discussing extraction methods, the ins and outs of LC/MS/MS, and hormone data.

I owe a debt of gratitude to my fellow graduate and undergraduate students who helped with sampling walrus bone, steroid hormone extractions, and discussion of data. Casey Clark, thank you for always taking the time to engage in meaningful discussions about my walrus data, help revise presentations, abstracts, and overall help in the project's sample collection and organization. I consider you an "honorary" committee member. Alex Thornton, thank you for coming onboard and taking part in the WALRUS project. The office chats were always helpful in relieving stress. The undergraduate students, Samuel George, Chase Markel, Eric Van Dam,

and Logan Wendling, thank you for your consistent and thorough work throughout this project. I would still be in the laboratory processing samples if you all were not onboard the project.

Lastly, I would like to thank my friends and family for always supporting me in my endeavors in marine mammal biology. My friends here at UAF, who all helped me take time to relax on this journey; it was great sharing beers, coffees, and bonfires with you all. Anastasia Brondino, my lovely girlfriend, for always telling me to push through, and that I could finish this project no matter what, but told me to relax on Sundays. To my mom and stepdad, Geralyn Charapata-Marsh and James Marsh, whose constant love and support confirmed to me that I was on the right path in life and to always follow my dreams. To all my siblings, Nathan, Frances, David, Aaron, and Nolan, who only gave me positive comments and thoughts throughout my whole undergraduate and graduate career and never told me to quit. My late father, Francis Charapata, who I know is proud of me and watched over me this entire time.

Chapter 1: General Introduction

The Pacific walrus (*Odobenus rosmarus divergens*, walrus hereafter) is an Arctic pinniped that relies on sea ice as a resting, migration, breeding, and feeding platform (Fay 1982). However, the current Arctic warming is substantially altering critical sea ice habitat of walruses. The stress response and reproductive status of the walrus to the current warming period in the Arctic is unknown. The objective of this study is to measure steroid hormone concentrations in walrus tissues (i.e., bone, blubber, and serum) to investigate if and how walrus stress response and reproductive status have changed over the past 3450 years. The selected time span includes previous warming and cooling periods in the Arctic, including the Medieval Warming period (approximately 1215 – 700 years before present (BP), Kinnard et al. 2011). We extracted steroid hormones from walrus bone collected from archaeological dig sites, museum collections, and subsistence harvests throughout the Alaskan and Russian Arctic. This type of study allows for the understanding of how walruses have fared through previous periods of change in the Arctic, and provide insight into how modern day walruses will physiologically respond to climate change. This information is critical to Native Alaskans, who rely on walruses for food, handicrafts, and whose culture has been influenced by these animals for thousands of years.

Walruses are highly associated with sea ice in both the winter and summer seasons (Fay 1982, Jay et al. 2012, 2014, Schonberg et al. 2014). Generally, during the winter seasons, sea ice forms as far south as 55 °N in the Bering Sea (Mahoney et al. 2011). Two main congregations of walruses form during the winter, one near St. Lawrence Island and the other in the Gulf of Anadyr, and breeding activity takes place throughout January and February (Fay 1982). In the spring, mature females and their calves, subadult males, and immature females migrate north toward the summer feeding grounds in the Chukchi Sea along the Alaska and Russian coasts

(Fay 1982, Sheffield and Grebmeier 2009, Jay et al. 2012). Mature males stay in the Bering Sea and congregate on haulouts in Bristol Bay near Round Island and the Gulf of Anadyr (Fay 1982). Important summer feeding areas have been identified for the walrus population, specifically for females and their calves, which include the high benthic biomass areas of the northeastern Chukchi Sea (i.e., Hanna Shoals area, Fay 1982, Jay et al. 2012, Schonberg et al. 2014). The majority of the walrus population feeds in the Chukchi Sea until around October when pack ice begins to form (Fay 1982). Following the formation of sea ice with the onset of winter, walruses in the Chukchi Sea and mature males from Bristol Bay and the Gulf of Anadyr proceed to the breeding grounds in the northern Bering Sea (Fay 1982).

In recent years, the Arctic summer sea ice cover and thickness has decreased dramatically and will likely continue to do so (Mahoney et al. 2011, IPCC 2013). If sea ice recedes from the Chukchi and Bering Sea shelves and retreats farther offshore over the deeper Arctic basin, walruses cannot take advantage of their benthic prey, as they are limited by their maximum dive depth of about 250 m (Fay 1982, Fay and Burns 1988, Jay and Fischbach 2008, Jay et al. 2012). Decreased sea ice also has an impact on Arctic ecosystems in general, and several scenarios affecting walrus ecosystems are possible. The Arctic shelf system could switch from a benthic dominated food web to a pelagic dominated system due to changes in sea ice-derived primary production and efficiency of pelagic trophic pathways (Grebmeier et al. 2006, Bluhm and Gradinger 2008, Grebmeier 2012). One walrus needs to consume the caloric equivalent of approximately 3000 - 6000 large clams/day equaling an estimated 16359 - 68960 kcal/day (e.g., *Serripes* spp., *Mya* spp., Noren et al. 2012). The switch to an Arctic system more dominated by pelagic production could potentially alter benthic prey abundance, and thus walrus foraging ecology.

Walruses may also feed on pelagic prey, such as ice seals (Lowry and Fay 1984, Sheffield and Grebmeier 2009), and it has been estimated that 1/3 of a ringed seal (*Pusa hispida*) is the energetic equivalent of the daily bivalve intake of a walrus (Seymour et al. 2014a). A shift in food web structure to a more pelagically dominated system could thus increase reliance of walruses on seals and other pelagic prey *in lieu* of benthic organisms. A switch to predominantly higher trophic level prey has not yet emerged within the past ten years (Seymour et al. 2014b), but such a switch could cause negative impacts on the body condition of walruses due to increased handling and digestive costs (Parker et al. 2009, Seymour et al. 2014a).

Current terrestrial haulouts of walruses (up to around ~35000 animals) on the Alaska coast have been reoccurring over the past ten years, and have also been attributed to a lack of sea ice (Jay et al. 2012, Kryukova et al. 2014). Thus, walruses have been observed either foraging near coastal haulouts where benthic prey may not be as abundant, or travelling long distances (~200 km roundtrip) to Hanna Shoal (Jay et al. 2012, Schonberg et al. 2014). Walruses could be utilizing body reserves on these long trips from land when in the past, sea ice would have provided a platform with easy access. If body reserves of walruses decline, negative effects on reproductive success can occur, and have already been documented (Garlich-Miller et al. 2006), that can eventually affect the population as a whole (Burek et al. 2008).

Compared with other pinnipeds, walruses have a long gestation period of around 15 months (11 months for harbor seals, *Phoca vitulina*, and California sea lions, *Zalophus californianus*, Pomeroy 2011). Walrus females are diestrus and become receptive for fertilization by males in early December and August (Fay 1982). Females also ovulate multiple times during an estrus period (Born 2001). However, walrus females are functionally monoestrus, as sexually mature males only produce viable sperm from December to March (Fay 1982). The late estrus

period (August) has been attributed to the process of sexual maturity, where females are experiencing "pseudo-pregnancies" in which a corpus luteum (ovarian endocrine tissue) is formed, but pregnancy does not occur (Fay 1982, Born 2001, Pomeroy 2011). When mating does occur (December-March), implantation of the blastocyst can be delayed for up to 5 months, with most implantations occurring from June to July followed by parturition from mid-April to mid-June of the following year (Fay 1982).

The calving interval for walruses is usually around 2 to 3 years, and calves leave their mothers before another calf is born, which means that females can lactate for over two years (Fay 1982, Pomeroy 2011). Females are highly protective of their offspring, and female/calf pairs form "nursery herds" that include up to 200 females (Fay 1982). While other pinnipeds mothers leave their pups to feed, which can lead to abandonment, walrus calves never leave their mothers (Fay 1982, Jemison and Kelly 2001). However, with the recent rapid loss of sea ice during the spring migrations to the summer feeding grounds, sightings of abandoned walrus calves have increased (Cooper et al. 2006, Metcalf and Robards 2008). In addition, Native subsistence hunters who rely on walruses for food, economic, and cultural purposes, have reported female walruses arriving at Wrangel Island in poor body condition (Metcalf and Robards 2008). Disturbances to the recently observed extensive terrestrial haulouts have led to an increase in calf mortality due to trampling, further reducing calf recruitment and adding to the difficulty in maintaining a healthy walrus population (Udevitz et al. 2013). Thus, without sea ice as a resting platform during migrations, and reduced benthic prey, longer travelling distances during migrations will have a greater effect on pregnant and lactating females. These effects of reduced sea ice on pregnant and lactating females, who require a higher caloric intake (Noren et al. 2014), could reduce walrus fecundity. The reduced size and fecundity of female Steller sea

lions (*Eumetopias jubatus*) in Alaska (Calkins et al. 1998, Pitcher et al. 1998, Trites and Donnelly 2003) has also been attributed to this type of nutritional stress (i.e., reduction of preferred prey), which in turn has contributed to a significant decline in parts of the Steller sea lion population (Trites and Donnelly 2003). Due to reduced sea ice, walruses also face possible nutritional stress and increases in energy expenditure, leading to a possible decline in fecundity and overall population size (Grebmeier et al. 2006, Garlich-Miller et al. 2006, Bluhm and Grandinger 2008, Jay et al. 2012, MacCracken 2012). Thus, walruses have been listed as a candidate for the Endangered Species Act, with a final decision of listing or not listing forthcoming in 2017 (USFWS 2011). This clearly illustrates a critical need to better understand the adaptive ability and direction of response by walruses facing current climatic changes.

1.1 Role of Steroid Hormones in Marine Mammal Physiology

Steroid hormones are lipid-based chemical messengers synthesized from cholesterol and have been used as a tool for studying the stress response and reproductive status in various marine mammals (Norris 1997, Amaral 2010). Glucocorticoids are a subclass of corticosteroid hormones that include cortisol (Norris 1997). When an animal exhibits a stress response to a stimulus (e.g., encountering a predator), the pars distalis (a portion of the lower brain) releases adrenocorticotropic hormone (ACTH), which signals the adrenal cortex to synthesize glucocorticoids (e.g., cortisol, Norris 1997). Cortisol is then released into the blood stream, attaches to plasma-binding proteins in the blood (steroid hormones not attached to proteins can be destroyed by kidney and liver activity), and is transported throughout the body (Norris 1997). Cortisol helps an animal cope with nutritional stress by metabolizing fat stores, conserving blood glucose levels, and increasing circulating fatty acids (Norris 1997, Peckett et al. 2011, Kershaw and Hall 2016). Cortisol also suppresses the immune system which is beneficial when the acute

stressor is not a disease but a situation such as predator avoidance, in which the immune system is not needed (Sapolsky et al. 2000). Once the stress response is complete, cortisol levels drop due to a direct negative feedback loop relating to ACTH levels (Norris 1997). However, under situations of chronic stress such as starvation, this negative feedback loop is weak and cortisol levels remain high resulting in negative impacts on the health of the animal (Norris 1997, Gulland et al. 1999, Burek et al. 2008, Kershaw and Hall 2016). The constant elevated glucocorticoids (e.g., cortisol) during chronic nutritional stress promote lipolysis of blubber stores and the catabolizing of muscle tissue for gluconeogenesis, reducing blubber stores and muscle mass (Norris 1997, Sapolsky et al. 2000). Chronic nutritional stress has been associated with reduced body size and fecundity in female Steller sea lions (Calkins et al. 1998, Pitcher et al. 1998, Trites and Donnelly 2003). Constant elevated cortisol levels in harbor seals were related to immune suppression and resulted in higher susceptibility to diseases (Gulland et al. 1999, Burek et al. 2008).

Progesterone, testosterone, and estradiol are reproductive steroid hormones used to monitor the reproductive status of marine mammals (reviewed in Amaral 2010). Similar to cortisol, the pars distalis is responsible for secreting the follicle-stimulating hormone (FSH) and the luteinizing hormone (LTH), which target the gonads of both males (testes) and females (ovaries), stimulating the production of testosterone in males and estradiol and progesterone in females (Norris 1997). Prior to ovulation, estradiol is secreted by the ovaries to prepare the uterus lining for implantation of the embryo, and stimulates the growing follicle which initiates estrus in females (Norris 1997, Pomeroy 2011). Once the follicle ruptures and the oocyte is released, the follicle grows into the corpus luteum (Pomeroy 2011). If fertilization occurs, the corpus luteum produces progesterone and estradiol to maintain the vascularized uterine lining for

implantation of the fertilized ovum and maintenance of the growing embryo (Norris 1997). In males, estradiol plays a role in maintaining fluid reabsorption in the testes, which helps produce viable sperm (Hess 2003). Progesterone in males initiates male sexual behavior by providing a precursor to testosterone when stress inhibits natural circulating testosterone concentrations (Wagner 2006). In males, testosterone is produced by the Leydig cells of the testes, and stimulates meiosis in primary spermatocytes (Norris 1997). High levels of testosterone in females correlate with receptivity to males, dominance among females, and pregnancy (Beehner et al. 2005, Welling et al. 2007).

Stress (cortisol) and reproductive (estradiol, progesterone, and testosterone) steroid hormones have been used to study the physiology of walruses (Tryland et al. 2009, Kinoshita et al. 2012, Muraco et al. 2012, Triggs 2013, Seymour 2014), as well as other pinnipeds (reviewed in Amaral 2010, Harcourt et al. 2010, Pomeroy 2011, Kershaw and Hall 2016) and cetaceans (reviewed in Amaral 2010, Pérez et al. 2011, Pomeroy 2011, Trego et al. 2013, Kellar et al. 2009, 2013, 2015, Hunt et al. 2014, Trana et al. 2015, Vu et al. 2015). However, commonly used tissues (feces, urine, serum, and blubber, Amaral 2010) that are collected to monitor the stress response and reproductive status of modern walruses and other marine mammal populations have not been archived before the onset of the current Arctic warming (IPCC 2013).

1.2 History of Climate Change in the Arctic and Steroid Hormones in Bone

The Arctic has experienced warming and cooling periods throughout the past 10000 years. Starting with the early Holocene period (11000 calendar years before present, hereafter abbreviated BP), proxy records indicate that temperatures were higher and less sea ice was present compared with today's climate (Polyak et al. 2009). This is true for the Chukchi Sea in the Arctic as well (De Vernal et al. 2005). After this warm period (~11000 – 7000 BP), a period

of cooling, referred to as Neoglacial cooling, began and culminated with the Little Ice Age occurring from 600 – 100 BP (Overpeck et al. 1997, Dyke et al. 1999, Polyak et al. 2009). However, there were also natural warming periods throughout this overall gradual cooling period, such as the Medieval Warming period that occurred prior to the Little Ice Age (1415 – 615 BP, Cronin and Cronin 2015). Thus, walruses have survived previous periods of warming (and cooling) in the Arctic, and it has been theorized that their range may have always been dependent on seasonal sea ice patterns during these prehistoric times (Dyke et al. 1999, Garlich-Miller et al. 2011). The Arctic has undergone a cooling period over the past 2000 years, however this cooling period has recently been reversed when four of the five warmest decades occurred from 1950 – 2000, coinciding with anthropogenic warming (Kaufman et al. 2009). The reversal of the Arctic cooling period is possibly correlated with an influx of the warmest Atlantic water on record (over the past 2000 years) arriving in the Arctic, which is linked to the currently observed reduction in sea ice in the circumpolar Arctic (Spielhagen et al. 2011).The current rate of sea ice loss is unprecedented over the past 1450 years based on terrestrial and oceanic proxies for sea ice extent including tree-ring chronologies and ice core records, respectively (Kinnard et al. 2011, IPCC 2013). Walruses have been restricted to coastal haulouts, due to lack of sea ice over the Arctic shelves, for about a decade (Jay et al. 2012). Thus, comparing the stress response and reproductive status of walruses from archaeological times, before the onset of the industrial revolution in 1750 (IPCC 2013), with those of modern walruses would effectively determine the physiological resilience of walruses in the face of climate change.

Hard parts of an animal such as bone and keratinized tissues, remain intact over thousands of years, providing a possible matrix for "true" sampling of walrus stress response and reproductive status baseline. Recent advances in forensic science make it possible to extract

steroid hormones including cortisol, as a bioindicator of stress response, as well as reproductive hormones from archaeological mammal bone (Mark et al. 2011). Reproductive hormones have also been extracted from modern rat (*Rattus* spp.) bone (Yarrow et al. 2010). Influences from outside soils and degradation do not significantly affect the composition and preservation of lipids in bones, although minor lipid degradation is possible (Evershed et al. 1995). Steroid hormones are lipophilic, preserved in ancient bone (Mark et al. 2011), and have a slow turnover rate in cortical bone (3 % / year in humans, Clarke 2008), making bone an ideal substrate to monitor long-term changes in stress response (cortisol concentrations) and reproductive status (estradiol, progesterone, and testosterone concentrations) of walruses. By detecting changes in stress and reproductive hormone concentrations in well preserved archaeological bone, archived bone from museum collections, and modern walrus bone from subsistence hunters, a novel tool comparing the baseline physiological status (in this case stress response and reproductive status) of archaeological walruses with present day walruses can be developed. However, analysis of steroid hormones extracted from mammal bone is still in its infancy, and until this study, they had not yet been extracted from ancient or modern marine mammal bone.

1.3 Contents of Thesis

The overall goal of this thesis was to understand the physiological resilience of the Pacific walrus to climate change in the Arctic by analyzing how stress and reproductive steroid hormone concentrations have changed over the past 3450 years in walrus bone. We first needed to better understand how bone steroid hormones relate to steroid hormones measured in blubber and serum because they are commonly used tissues in marine mammal steroid hormone studies (e.g., Myers et al. 2010, Kellar et al. 2013). Thus, chapter 2: "A tissue comparison of steroid hormones in bone, serum, and blubber of Pacific walruses", investigates the differences in

cortisol, estradiol, progesterone, and testosterone concentrations in bone relative to blubber and serum. In addition, correlations among bone, blubber, and serum are analyzed to determine if blubber or serum steroid hormone concentrations can predict bone steroid hormone concentrations in walruses. These correlations will allow for continued monitoring of the current walrus population through periods of environmental change using minimally invasive techniques like blubber biopsies that can be related to established baselines prior to the industrial revolution.

Chapter 3, "Steroid hormone concentrations in Pacific walrus bone reveal long-term changes in reproductive status and stress response over the last 3 millennia" investigates the extraction and validation of steroid hormone concentrations (cortisol, estradiol, progesterone, and testosterone) in walrus bone from archaeological (> 200 BP), historical (200 – 20 BP), and modern time periods (2014 – 2015). In addition, stress response and reproductive status of walruses from archaeological time periods are compared with present day walruses. Decadal patterns of walrus stress response and reproductive status from the past 180 years are also analyzed. Both chapters provide insights into the physiological resiliency of walruses in a rapidly changing Arctic, and offer a better understanding of a novel tool (bone steroid hormones) used for long-term monitoring of stress and reproductive status of walruses.

1.4 References

Amaral, R. 2010. Use of alternative matrices to monitor steroid hormones in aquatic mammals: A review. Aquatic Mammals 36:162–171.

Beehner, J. C., J. E. Phillips-Conroy, and P. L. Whitten. 2005. Female testosterone, dominance rank, and aggression in an Ethiopian population of hybrid baboons. American Journal of Primatology 67:101–119.

Bluhm, B., and R. Gradinger. 2008. Regional variability in food availability for Arctic marine mammals. Ecological Applications 18:77–96.

Born, E. W. 2001. Reproduction in female Atlantic walruses (*Odobenus rosmarus rosmarus*) from north-west Greenland. Journal of Zoology 255:165–174.

Burek, K. A., F. M. D. Gulland, and T. M. O'Hara. 2008. Effects of climate change on Arctic marine mammal health. Ecological Applications 18:126–134.

Calkins, D. G., E. F. Becker, and K. W. Pitcher. 1998. Reduced body size of female Steller sea lions from a declining population in the Gulf of Alaska. Marine Mammal Science 14:232–244.

Clarke, B. 2008. Normal bone anatomy and physiology. Clinical Journal of the American Society of Nephrology 3:131–139.

Cooper, L. W., C. J. Ashjian, S. L. Smith, L. A. Codispoti, J. M. Grebmeier, R. G. Campbell, and E. B. Sherr. 2006. Rapid seasonal sea-ice retreat in the Arctic could be affecting Pacific walrus (*Odobenus rosmarus divergens*) recruitment. Aquatic Mammals 32:98–102.

Cronin, T. M., and M. A. Cronin. 2015. Biological response to climate change in the Arctic Ocean: the view from the past. Arktos 1:1–18.

De Vernal, A., C. Hillaire-Marcel, and D. A. Darby. 2005. Variability of sea ice cover in the Chukchi Sea (western Arctic Ocean) during the Holocene. Paleoceanography doi:10.1029/2005PA001157

Dyke, A. S., J. Hooper, C. R. Harington, and J. M. Savelle. 1999. The late Wisconsinan and Holocene record of walrus (*Odobenus rosmarus*) from North America: A review with new data from Arctic and Atantic Canada. Arctic Institute of North America 52:160–181.

Evershed, R. P., G. Turner-Walker, R. E. M. Hedges, N. Tuross, and A. Leyden. 1995. Preliminary results for the analysis of lipids in ancient bone. Journal of Archaeological Science 22:277–290.

Fay, F. H. 1982. Ecology and biology of the Pacific walrus, *Odobenus rosmarus divergens* Illiger. North American Fauna 74:1–266.

Fay, F. H., and J. J. Burns. 1988. Maximal feeding depth of walruses. Arctic 41:239–240.

Garlich-Miller, J. L., L. T. Quakenbush, and J. F. Bromaghin. 2006. Trends in age structure and productivity of Pacific walruses harvested in the Bering Strait region of Alaska, 1952-2002. Marine Mammal Science 22:880–896.

Garlich-Miller, J. L., J. G. MacCracken, J. Snyder, R. Meehan, M. Myers, J. M. Wilder, E. Lance, and A. Matz. 2011. Status review of the Pacific walrus (*Odobenus rosmarus divergens*). U.S. Fish and Wildlife Service, Marine Mammals Management, Anchorage, AK.

Grebmeier, J. M., L. W. Cooper, H. M. Feder, and B. I. Sirenko. 2006. Ecosystem dynamics of the Pacific-influenced northern Bering and Chukchi Seas in the Amerasian Arctic. Progress in Oceanography 71:331–361.

Grebmeier, J. M. 2012. Shifting patterns of life in the pacific Arctic and sub-Arctic Seas. Annual Review of Marine Science 4:63–78.

Gulland, F. M. D., M. Haulena, and B. Hilliard. 1999. Adrenal function in wild and rehabilitated Pacific harbor seals (*Phoca vitulina richardsii*). Marine Mammal Science 15:810–817.

Harcourt, R. G., E. Turner, A. Hall, J. R. Waas, and M. Hindell. 2010. Effects of capture stress on free-ranging, reproductively active male Weddell seals. Journal of Comparative Physiology A 196:147–154.

Hess, R. A. 2003. Estrogen in the adult male reproductive tract: A review. Reproductive Biology and Endocrinology 1:52.

Hunt, K. E., R. Stimmelmayr, C. George, C. Hanns, R. Suydam, H. Brower Jr, and R. M. Rolland. 2014. Baleen hormones: a novel tool for retrospective assessment of stress and reproduction in bowhead whales (*Balaena mysticetus*). Conservation Physiology 2:1–12.

IPCC. 2013. Climate Change 2013: The physical science basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. T. F. Stocker, D. Qin, G. K. Plattner, M. Tignor, S. K. Allen, J. Boschung, K. Nauels, Y. Xia, V. Bex, and P. M. Midgley, editors. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Jay, C. V., and A.S. Fischbach. 2008. Pacific walrus response to Arctic Sea ice losses. U.S. Geological Survey, Fact Sheet 2008–3041. 4 pp.

Jay, C. V., A. S. Fischbach, and A. A. Kochnev. 2012. Walrus areas of use in the Chukchi Sea during sparse sea ice cover. Marine Ecology Progress Series 468:1–13.

Jay, C. V., J. M. Grebmeier, A. S. Fischbach, T. L. McDonald, L. W. Cooper, and F. Hornsby. 2014. Pacific walrus (*Odobenus rosmarus divergens*) resource selection in the Northern Bering Sea. PloS one 9:e93035.

Jemison, L., and B. Kelly. 2001. Pupping phenology and demography of harbor seals (*Phoca Vitulina Richardsi*) on Tugidak Island, Alaska. Marine Mammal Science 17:585–600.

Kaufman, D. S., D. P. Schneider, N. P. Mckay, C. M. Ammann, R. S. Bradley, K. R. Briffa, G. H. Miller, B. L. Otto-Bliesner, J. T. Overpeck, B. M. Vinther, A. Lakes, and P. Members. 2009. Recent warming reverses long-term Arctic cooling. Science 325:4–7.

Kellar, N. M., M. L. Trego, C. I. Marks, S. J. Chivers, K. Danil, and F. I. Archer. 2009. Blubber testosterone: A potential marker of male reproductive status in short-beaked common dolphins. Marine Mammal Science 25:507–522.

Kellar, N. M., J. Keliher, M. L. Trego, K. N. Catelani, C. Hanns, J. C. George, and C. Rosa. 2013. Variation of bowhead whale progesterone concentrations across demographic groups and sample matrices. Endangered Species Research 22:61–72.

Kellar, N. M., K. N. Catelani, M. N. Robbins, M. L. Trego, C. D. Allen, K. Danil, and S. J. Chivers. 2015. Blubber cortisol: A potential tool for assessing stress response in free-ranging dolphins without effects due to sampling. PLoS ONE 10:1–16.

Kershaw, J. L., and A. J. Hall. 2016. Seasonal variation in harbour seal (*Phoca vitulina*) blubber cortisol - A novel indicator of physiological state? Scientific Reports 6:21889.

Kinnard, C., C. M. Zdanowicz, D. A. Fisher, E. Isaksson, A. De Vernal, and L. G. Thompson. 2011. Reconstructed changes in Arctic sea ice over the past 1,450 years. Nature 479:509–512.

Kinoshita, K., M. Kiwata, R. Kuwano, N. Sato, T. Tanaka, M. Nagata, H. Taira, and H. Kusunoki. 2012. Temporal association of serum progesterone concentrations and vaginal cytology in walruses (*Odobenus rosmarus*). Theriogenology 77:933–939.

Kryukova, N. V, A. A. Kochnev, and A. A. Pereverzev. 2014. The influence of ice conditions on terrestrial haulouts of the pacific walrus *Odobenus rosmarus divergens* Illiger, 1815 in the Gulf of Anadyr, Bering Sea. Russian Journal of Marine Biology 40:30–35.

Lowry, L. F., and F. H. Fay. 1984. Seal eating by walruses in the Bering and Chukchi Seas. Polar Biology 3:11–18.

MacCracken, J. G. 2012. Pacific walrus and climate change: observations and predictions. Ecology and Evolution 2:2072–2090.

Mahoney, A. R., J. R. Bockstoce, D. B. Botkin, H. Eicken, and R. A. Nisbet. 2011. Sea-ice distribution in the Bering and Chukchi seas: Information from historical Whaleships' logbooks and journals. Arctic 64:465–477.

Mark, L., Z. Patonai, A. Vaczy, N. Kajsza, and A. Marcsik. 2011. Hormone mass fingerprinting: novel molecular sex determination of ancient human skeletal remains. Pages 411–415 *in* I. Turbanti-Memmi, editor. Proceedings of the 37th International Symposium on Archaeometry; 12th-16th May, 2008, Siena, Italy. Springer-Verlag, Berlin, Heidelberg.

Metcalf, V., and M. Robards. 2008. Sustaining a healthy human-walrus relationship in a dynamic environment: challenges for comanagement. Ecological Applications 18:148–156.

Muraco, H. S., L. D. Coombs, D. G. Procter, P. J. Turek, and M. J. Muraco. 2012. Use of human chorionic gonadotropin in a male Pacific walrus (*Odobenus rosmarus divergens*) to induce rut and achieve a pregnancy in a nulliparous female. Journal of Andrology 33:789–97.

Myers, M. J., B. Litz, and S. Atkinson. 2010. The effects of age, sex, season and geographic region on circulating serum cortisol concentrations in threatened and endangered Steller sea lions (Eumetopias jubatus). General and Comparative Endocrinology 165:72–77.

Noren, S. R., M. S. Udevitz, and C. V. Jay. 2012. Bioenergetics model for estimating food requirements of female Pacific walruses *Odobenus rosmarus divergens*. Marine Ecology Progress Series 460:261–275.

Noren, S. R., M. S. Udevitz, and C. V Jay. 2014. Energy demands for maintenance, growth, pregnancy, and lactation of female Pacific walruses (*Odobenus rosmarus divergens*). Physiological and Biochemical Zoology 87:837–54.

Norris, D. 1997. Vertebrate Endocrinology, 3rd ed. Academic Press, San Diego, California.

Overpeck, J., K. Hughen, D. Hardy, R. Bradley, R. Case, M. Douglas, B. Finney, K. Gajewski, G. Jacoby, A. Jennings, S. Lamoureux, A. Lasca, G. MacDonald, J. Moore, M. Retelle, S. Smith, A. Wolfe, and G. Zielinski. 1997. Arctic environmental change of the last four centuries. Science 278:1251–1256.

Parker, K. L., P. S. Barboza, and P. G. Michael. 2009. Nutrition integrates environmental responses of ungulates. Functional Ecology 23:57–69.

Peckett, A. J., D. C. Wright, and M. C. Riddell. 2011. The effects of glucocorticoids on adipose tissue lipid metabolism. Metabolism: Clinical and Experimental 60:1500–1510.

Pérez, S., Á. García-López, R. Stephanis, J. Giménez, S. García-Tiscar, P. Verborgh, J. M. Mancera, and G. Martínez-Rodriguez. 2011. Use of blubber levels of progesterone to determine pregnancy in free-ranging live cetaceans. Marine Biology 158:1677–1680.

Pitcher, K., D. Calkins, and G. Pendleton. 1998. Reproductive performance of female Steller sea lions: an energetics-based reproductive strategy? Canadian Journal of Zoology 76:2075–2083.

Polyak, L., J.T. Andrews, J. Brigham-Grette, D. Darby, A. Dyke, S. Funder, M. Holland, A. Jennings, J. Savelle, M. Serreze, and E. Wolff. 2009. History of Arctic sea ice *in* Past Climate Variability and Change in the Arctic and at High Latitudes. A report by the U.S. Climate Change Science Program and Subcommittee on Global Change Research. U.S. Geological Survey, Reston, VA. pp 159–184.

Pomeroy, P. 2011. Reproductive cycles of marine mammals. Animal Reproduction Science 124:184–193.

Sapolsky, R., L. Romero, and A. Munck. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews 21:55–89.

Schonberg, S. V., J. T. Clarke, and K. H. Dunton. 2014. Distribution, abundance, biomass and diversity of benthic infauna in the Northeast Chukchi Sea, Alaska: Relation to environmental variables and marine mammals. Deep-Sea Research Part II: Topical Studies in Oceanography 102:144–163.

Seymour, J., L. Horstmann-Dehn, and M. J. Wooller. 2014a. Proportion of higher trophic-level prey in the diet of Pacific walruses (*Odobenus rosmarus divergens*). Polar Biology 37:941–952.

Seymour, J., L. Horstmann-Dehn, and M. J. Wooller. 2014b. Inter-annual variability in the proportional contribution of higher trophic levels to the diet of Pacific walruses. Polar Biology 37:597–609.

Seymour, J. 2014. Pacific walrus use of higher trophic level prey and the relation to sea ice extent, body condition, and trichinellosis. Dissertation. University of Alaska Fairbanks, Fairbanks, AK.

Sheffield, G., and J. M. Grebmeier. 2009. Pacific walrus (*Odobenus rosmarus divergens*): differential prey digestion and diet. Marine Mammal Science 25:761–777.

Spielhagen, R., K. Werner, S. Sørensen, K. Zamelczyk, E. Kandiano, G. Budeus, K. Husum, T. Marchitto, and M. Hald. 2011. Enhanced modern heat transfer to the Arctic by warm Atlantic water. Science 331:450–453.

Trana, M. R., J. D. Roth, G. T. Tomy, W. G. Anderson, and S. H. Ferguson. 2015. Increased blubber cortisol in ice-entrapped beluga whales (*Delphinapterus leucas*). Journal of Experimental Marine Biology and Ecology 462:8–13.

Trego, M. L., N. M. Kellar, and K. Danil. 2013. Validation of blubber progesterone concentrations for pregnancy determination in three dolphin species and a porpoise. PLoS One 8:e69709.

Triggs, L. 2013. Establishing endocrine and behavioral parameters of reproduction in Pacific walrus. Thesis. University of Washington, Seattle, WA.

Trites, A. W., and C. P. Donnelly. 2003. The decline of Steller sea lions *Eumetopias jubatus* in Alaska: a review of the nutritional stress hypothesis. Mammal Review 33:3–28.

Tryland, M., C. Lydersen, K. M. Kovacs, and S. I. Thoresen. 2009. Serum chemistry reference values in free-ranging North Atlantic male walruses (*Odobenus rosmarus rosmarus*) from the Svalbard archipelago. Veterinary Clinical Pathology 38:501–506.

Udevitz, M. S., R. L. Taylor, J. L. Garlich-Miller, L. T. Quakenbush, and J. A. Snyder. 2013. Potential population-level effects of increased haulout-related mortality of Pacific walrus calves. Polar Biology 36:291–298.

U.S. Fish and Wildlife Service (USFWS). 2011. Pacific walrus to be designated a candidate for endangered species protection. Available at http://www.fws.gov/news/ShowNews.cfm?ID==33022651-D390-154B-CDD13E0723B3C928 (accessed March 11, 2016)

Vu, E. T., C. Clark, K. Catelani, N. M. Kellar, and J. Calambokidis. 2015. Seasonal blubber testosterone concentrations of male humpback whales (*Megaptera novaeangliae*). Marine Mammal Science 31:1258–1264.

Wagner, C. K. 2006. The many faces of progesterone: A role in adult and developing male brain. Frontiers in Neuroendocrinology 27:340–359.

Welling, L. L. M., B. C. Jones, L. M. DeBruine, C. A. Conway, M. J. Law Smith, A. C. Little, D. R. Feinberg, M. A. Sharp, and E. A. S. Al-Dujaili. 2007. Raised salivary testosterone in women is associated with increased attraction to masculine faces. Hormones and Behavior 52:156–161.

Yarrow, J. F., C. F. Conover, J. A. Lipinska, C. A. Santillana, T. J. Wronski, and S. E. Borst. 2010. Methods to quantify sex steroid hormones in bone: applications to the study of androgen ablation and administration. American Journal of Physiology - Endocrinology and Metabolism 299:E841–E847.

Chapter 2: A Tissue Comparison of Steroid Hormones in Bone, Serum, and Blubber of Pacific Walruses [1]

2.1 Abstract

Steroid hormones from archived bone samples could provide an opportunity to capture baseline stress responses (*i.e.*, cortisol) and reproductive status (*i.e.*, estradiol, progesterone, and testosterone) of the Pacific walrus (*Odobenus rosmarus divergens*) prior to the reduction of their sea ice habitat due to the current climate warming in the Arctic. However, bone is not commonly used in steroid hormone studies and is difficult to acquire, as animals must be deceased. In this study, the relationships of hormone concentrations (cortisol, estradiol, progesterone, and testosterone) between bone, blubber, and serum were examined from individual adult walruses (n = 34) harvested by Native Alaskan subsistence hunters during 2014 and 2015. Bone cortisol concentrations were similar to blubber (P = 0.96), but significantly lower compared with serum (P = < 0.001). Bone estradiol concentrations from 2014 were significantly higher than estradiol concentrations from 2015 (P = < 0.001). Significant interannual differences of steroid hormones from bone were only exhibited in estradiol. Thus, estradiol may have a different reservoir time in cortical bone compared with other steroid hormones measured in this study. Bone progesterone concentrations were significantly different between sexes and highly dependent on the sex sampled when significant differences were present among tissues (P = < 0.001, 0.009, sexes, and sex*tissue, respectively). Bone testosterone concentrations were significantly higher than

[1] Submitted to Marine Mammal Science as Charapata P, Horstmann L, Misarti N "A tissue comparison of steroid hormones in bone, serum, and blubber of Pacific walruses"

blubber ($P = 0.003$), but similar to serum concentrations ($P = 0.26$). Linear regression analysis revealed a significant positive correlation among bone and blubber progesterone concentrations ($P = < 0.001$, $R^2 = 0.51$) in males. In females, bone and blubber progesterone concentrations exhibited a strong, but not significant correlation ($P = 0.06$, $R^2 = 0.75$), which is likely the result of small sample size ($n = 5$). Our results provide the first stress response and reproductive status in free-ranging walruses *via* steroid hormone analysis of bone, blubber, and serum. Specifically, estradiol measured in bone has a different reservoir time compared with other hormones measured in this study. This finding should be considered when interpreting estradiol concentrations measured in bone. In addition, progesterone from blubber samples is comparable with bone progesterone concentrations, allowing monitoring of walrus reproductive status using more easily obtainable tissues compared with bone.

2.2 Introduction

The Pacific walrus (*Odobenus rosmarus divergens*) (hereafter referred to as walrus) is currently a candidate for the Endangered Species Act, and a decision on whether they should be listed will be made by 2017 (USFWS 2014). Current Arctic warming and rapid recession of critical summer sea ice habitat are prompting concerns for ice-adapted species, such as walruses that use ice for feeding, molting, breeding, and resting (Fay 1982, IPCC 2013, USFWS 2014). Walruses are benthic feeders, primarily preying on bivalves, and thus forage in shallow (~250m) benthic habitats (*e.g.*, Hannah Shoals, Fay 1982, Fay and Burns 1988). Walruses are able to conserve energy while foraging by utilizing sea ice as a floating access point to various feeding areas (Fay 1982). However, with the current recession of summer sea ice into the Arctic basin (*i.e.*, depths > 250 m), walruses are restricted to land haulouts and must travel substantial distances to access their primary summer feeding grounds or forage in less productive coastal areas (Jay *et al.* 2012). In addition, rapid recession of sea ice has also resulted in separation of mothers and calves during their spring migration to summer feeding grounds (Cooper *et al.* 2006), and trampling of calves while utilizing land haulouts (Udevitz *et al.* 2013). Walruses have been experiencing a decline in fecundity since the late 1980s (Garlich-Miller *et al.* 2006), and with increases in calf mortality, the viability of a healthy reproductive walrus population may be in jeopardy. Future predictions call for further reduction of summer sea ice walrus habitat (Douglas 2010, MacCracken 2012), potentially resulting in northward shifts of summer prey resources due to changes in the food web and northward shifts of winter breeding grounds due to early sea ice retreat (Bluhm and Gradinger 2008, Grebmeier 2012, MacCracken 2012, Jay *et al.* 2014). Monitoring of physiological resilience (*i.e.*, stress response and reproductive status) of

walruses to their changing environment is imperative for the conservation of an important marine mammal that Native Alaskans rely on as a food, cultural, and economic resource (Metcalf and Robards 2008).

Stress (*i.e.*, cortisol) and reproductive (*i.e.*, estradiol, progesterone and testosterone) steroid hormones provide important physiological information about marine mammals. In mammals, cortisol is released from the adrenal cortex in response to a stimulus to prompt the mobilization of glucose from various tissues in aid of the "fight or flight" response (Norris 1997). However, under chronic release of cortisol, normal body function is impaired (Norris 1997). In pinnipeds, high cortisol concentrations have been linked to susceptibility to disease, lower body condition, and ultimately problems with reproduction (Gulland *et al.* 1999, Fair and Becker 2000, Burek *et al.* 2008). These effects of acute and chronically elevated cortisol concentrations result in a reliable biomarker of acute and chronic stress responses in pinnipeds (reviewed in Amaral 2010, Myers *et al.* 2010, Seymour 2014, Kershaw and Hall 2016). Estradiol, progesterone, and testosterone indicate reproductive status (*i.e.*, pregnancy and sexual maturity) and timing of the breeding seasons in pinnipeds (Atkinson and Gilmartin 1992, Bartsh *et al.* 1992, Pomeroy 2011, Kinoshita *et al.* 2012, Muraco *et al.* 2012, Zhang *et al.* 2014).

Steroid hormones have been extracted from a variety of marine mammal tissues, most commonly from serum and blubber (reviewed by Amaral 2010). Estradiol and progesterone extracted and measured in serum of captive female walruses (Kinoshita *et al.* 2012, Muraco *et al.* 2012, Triggs 2013) reflect changes in reproductive status. Serum testosterone measured in captive male walruses showed seasonal changes with reproductive behavior (Kinoshita *et al.* 2012, Muraco *et al.* 2012). This follows similar trends from studies on wild male pinnipeds,

where serum testosterone concentrations increased before and during natural breeding seasons and declined thereafter (Atkinson and Gilmartin 1992, Zhang *et al.* 2014). Cortisol has been measured in serum of free-ranging male Atlantic walruses (*Odobenus rosmarus rosmarus*, Tryland *et al.* 2009), and various other pinniped species, but seems to be an unreliable measure of baseline stress response due to the induced high cortisol concentrations during capture and handling of the animals (Myers *et al.* 2010, Zhang *et al.* 2014, Kershaw and Hall 2016).

Cortisol has been measured in blubber of free-ranging walruses (Seymour 2014) and harbor seals (*Phoca vitulina*, Kershaw and Hall 2016). Seymour (2014) showed walruses exhibited a wide range of blubber cortisol concentrations, and argued that blubber served as a multiple-month long-term reservoir of cortisol. In harbor seals, blubber cortisol was not affected by capture stress as seen in serum cortisol concentrations and significantly varied by sex and throughout molting and fasting periods (Kershaw and Hall 2016). In blubber, progesterone and testosterone have only been extracted from cetaceans and are a useful tool for determining reproductive status of females and males, respectively (Mansour *et al.* 2002, Kellar *et al.* 2006, 2009, 2013, Trego *et al.* 2013, Vu *et al.* 2015).

Serum has been associated with circulating concentrations of steroid hormones, while blubber is a long-term reservoir accumulating large pulses of steroid hormones originating from serum during pregnancy and molting events, and then equalizing with serum concentrations thereafter (Kellar *et al.* 2013, Kershaw and Hall 2016). However, while blubber and serum are used in many current studies, these tissues are not generally archived over the necessary time frames to capture baseline steroid hormone concentrations of walruses before the onset of

substantial anthropogenic activity in 1750, which has contributed to the current warming in the Arctic (IPCC 2013).

Bone contains lipids that do not degrade substantially after death of an animal, and have been detected and measured after bones had been buried for thousands of years (Evershed *et al.* 1995, During *et al.* 2015). Steroid hormones are lipophilic compounds, meaning they are highly associated with lipids (Norris 1997). Testosterone and estradiol have been extracted from both ancient (6831 years before present (BP)) human (Mark *et al.* 2011) and modern male F344 rat (*Rattus* sp.) bone (Yarrow *et al.* 2010). While the reservoir time of bone steroid hormones is unknown (Yarrow *et al.* 2010), bone has a slow turnover rate (3% cortical bone/year, Clarke 2008), making bone steroid hormone concentrations potentially an accumulated average over the lifetime of an animal. Thus, bone steroid hormone concentrations have the benefits of not being skewed by acute stress response and reproductive events compared with serum and blubber (Kellar *et al.* 2013, Kershaw and Hall 2016). In addition, steroid hormone concentrations extracted from archaeological (> 200 years BP) and historical (200 – 20 years BP) walrus bone housed in museum collections would represent an accurate baseline stress response and reproductive status of walruses prior to warming in the Arctic.

Significant warming in the Arctic has occurred since the 1950s, when an Arctic cooling period was reversed (Kaufman *et al.* 2009). Beginning in 2007, substantial reduction to the summer sea ice habitat has restricted portions of the walrus population to land haulouts (Jay and Fischbach 2008, Jay *et al.* 2012). Comparing walrus bone steroid hormone concentrations among archaeological and current time periods could give insight into the physiological resiliency of walruses to a changing environment. However, no studies have addressed how bone steroid

hormones relate to tissues that are regularly used in marine mammal studies, such as serum and blubber.

Use of novel tissues and matrices to extract steroid hormones has been increasing (Hogg *et al.* 2009, Kellar *et al.* 2013, 2015, Trumble *et al.* 2013, Hunt *et al.* 2014), yet, little is known about the interrelationship of steroid hormones among tissues. Kellar *et al.* (2013) compared progesterone concentrations from blubber, serum, and urine within the same individual bowhead whales (*Balaena mysticetus*), to determine if concentrations were correlated among tissue types. One of the main objectives of these authors was to determine if correlations from commonly used tissues (*e.g.,* urine and serum) could accurately estimate the progesterone concentration of a novel tissue (blubber) for the determination of reproductive status of bowhead whales (Kellar *et al.* 2013). In this study, we have taken a similar approach to determine if blubber and serum can predict bone steroid hormone concentrations. Bones collected from archaeological digs and archived in museums have the potential to complement blubber and serum hormone studies. Hormones extracted from bone could extend the physiological timeline of marine mammals further into the past, before the start of serum and blubber archives from stranding programs (Kellar *et al.* 2009) and biopsy sampling projects (Vu *et al.* 2015). In addition, blubber and serum are somewhat more easily obtainable through biopsies and live captures compared to bone samples, where an animal would have to be deceased. Thus, if blubber and/or serum hormone concentrations can be compared with bone steroid hormones, future blubber and/or serum hormones studies could be compared with the extended physiological timeline provided by bone steroid hormones.

Native Alaskan subsistence users have been collaborating with researchers since the early 1950s, providing harvest data and tissue samples to monitor the health of the Pacific walrus population (*e.g.*, Fay *et al.* 1997, Garlich-Miller *et al.* 2006). Various tissues are collected that are viable for monitoring the stress response and reproductive status of the walrus population using hormone concentrations from harvested individuals (*e.g.*, Kellar *et al.* 2013).

In this study, we measured stress and reproductive hormone concentrations in bone, blubber, and serum from harvested walruses in the communities of Gambell and Savoonga on St. Lawrence Island, Alaska (AK). We had two main objectives; 1) to extract, validate, and compare steroid hormone concentrations among bone, blubber, and serum tissues to give insight into stress response and reproductive physiology of free-ranging walruses, and 2) to determine if tissues commonly used in steroid hormone analyses, blubber and serum, can predict bone steroid hormone concentrations. The results from this study add to the sparse published literature on the hormonal reproductive physiology of walruses (Tryland *et al.* 2009, Kinoshita *et al.* 2012, Muraco *et al.* 2012). In addition, these are the first reported data determining differences in cortisol, estradiol, progesterone, and testosterone concentrations among different tissues and sexes from free-ranging walruses. Determination of these correlations among tissues, specifically bone, allows direct comparison of steroid hormone concentrations in the modern population to those from an archaeological baseline population.

2.3 Methods

2.3.1 Collection of Samples

Blubber, serum, and bone were collected from Native Alaskan subsistence harvests through an agreement with Native hunters, the Eskimo Walrus Commission, the Alaska

Department of Fish and Game (ADF&G), and the U.S. Fish and Wildlife Service (USFWS) during May 2014 and 2015 from the communities of Gambell and Savoonga on St. Lawrence Island, AK, USA (Figure 2.1). Full thickness blubber with skin and muscle attached, blood, and bone were collected from an area of the walrus body that the hunters deemed adequate for collection, generally sternal blubber and a flipper long bone. In addition, skull and mandible bone elements were collected from individual walruses (n = 7 pairs) archived in the Mammal Collection at the University of Alaska Museum (UAM). The paired bone samples were used for a pilot study to determine if steroid hormones extracted from different bone elements are comparable. 25 mL of blood was collected from each animal in Falcon tubes containing anti-coagulating glass beads (Market Lab Inc., MS4491). Blubber, blood, and bone samples were kept at ambient temperature (~ -12 °C) until hunters returned to town. Blood was centrifuged within 8 hrs of collection, and serum was collected and frozen at -20 °C. Samples were shipped frozen to the Marine Mammal laboratory at the University of Alaska Fairbanks (UAF) and immediately transferred to -80 °C until steroid hormone analysis. Tissue samples were transferred to UAF under a Letter of Authorization to Dr. L. Horstmann. Hunters recorded sex, age class, and reproductive information for harvested females including; presence of a fetus, calf, yearling, pregnancy status, and/or if females were lactating. An estimated age based on counts of cementum growth layers in the walrus teeth (Carroll *et al.* 2013) was available for a majority of the samples and was performed at Matson's Cementum Aging Laboratory in MT. A list of tissue samples with provenience data (*e.g.*, sex, age class, estimated age) are provided in Appendix 2.1.

We collected all three tissues from females (n = 5) and males (n = 15). In addition, bone and serum (but not blubber) were collected from one female and one male, and bone and blubber

(but not serum) were collected from an additional 12 males. Overall, our total sample size was *n* = 34, with 6 females and 28 males.

2.3.2 Steroid Hormone Extraction

2.3.2.1 Bone Samples

Sections of walrus bone were polished with a sanding drum attachment on a Dremel® 3000 drill to remove outside contaminants exposing clean areas of cortical bone. Approximately 1.5 g of cortical bone was removed for steroid hormone extraction using a Dremel drill with a diamond blade attachment. Pieces of bone were pulverized into powder using a Wig-L-Bug® and 0.2 - 0.3 g of bone powder was transferred to 2.8 mL ceramic bead homogenizer cryovials. Samples were homogenized, dry, on a Disruptor Genie® (Scientific Industries) for one minute. Samples were spiked with 100 ng of isotopically labeled internal standards (Sigma Aldrich) (ISTD), d_4-cortisol, $^{13}C_3$-testosterone-2, d_9-progesterone, and d_5-estradiol, for accurate hormone detection and validation during liquid chromatography tandem mass spectrometry (LC/MS/MS, Difrancesco *et al.* 2007, Zhang *et al.* 2009, Koal *et al.* 2012, Murtagh *et al.* 2013). Lipids were extracted from powdered bone by adding 1.460 mL of methanol (BDH®, Accorsi *et al.* 2008, Bryan *et al.* 2013, Hunt *et al.* 2014). Samples were homogenized for three minutes on a Disruptor Genie® and set on a rocking platform (VWR®; Model 100) for 24 hours. Samples were then centrifuged (Microfuge® 18 Centrifuge, Beckman Coulter™) at 12000 RPMs for 20 minutes. Supernatant from each sample was pipetted into glass vials, and remaining methanol was evaporated under nitrogen gas (N-EVAP™112, Organomation Associates, Inc.) leaving only lipids. Samples were then stored in a -80 °C freezer until shipped for LC/MS/MS analysis. Steroid hormone concentrations are reported as ng/g bone powder. In addition, for reference to

lipid-corrected bone steroid hormone concentrations from archaeological and historical time periods, median bone hormone concentrations are also reported as ng/g lipid in Table 2.1 and Table 2.2. Due to low available sample mass, bone samples were not run in duplicates.

2.3.2.2 Blubber Samples

The oxidized outer layer of walrus blubber from each full thickness slab was removed with sterilized individual razor blades exposing fresh blubber tissue. Two separate vertical strips of full thickness blubber weighing between 0.2 - 0.3 g were removed starting from below the skin and ending above the muscle and transferred to separate 2.8 mL ceramic bead homogenizer cryovials. Samples were homogenized, ISTD added, and lipids extracted with methanol as described above, except samples were vortexed for 8 minutes after methanol and ISTDs were added to samples. Sample extracts were stored in a -80 °C freezer until shipped for LC/MS/MS analysis. Blubber samples were run in duplicate with the average concentration (ng/g blubber) used for analysis.

2.3.2.3 Serum Samples

Serum was thawed and mixed before steroid hormone extractions. For each serum sample, 375 µL of serum was added to 2.8 mL ceramic bead cryovials. Samples were spiked with ISTD and extracted using methanol as described above for bone samples. Sample extracts were stored in a -80 °C freezer until shipped for LC/MS/MS analysis. Serum samples were run in duplicate with the average concentration (ng/mL serum) used for analysis.

2.3.3 LC/MS/MS Analysis of Steroid Hormones

Prior to analysis, each sample was reconstituted in 200 µL of methanol, and each sample was split into two equal aliquots and dried again using an Eppendorf-Vacufuge rotary evaporating device. The first aliquot of each extract was derivatized with dansyl chloride according to Zhang *et al.* (2009) just prior to LC/MS/MS analysis. To each sample, 20 µL of 10 mM Na_2CO_3 and 50 µL of freshly prepared dansyl chloride solution (3 mg/mL acetone) were added. The samples were heated at 60 °C for 10 minutes. Samples were transferred to autosampler vials and immediately analyzed. The second aliquot of each extract was derivatized with the AB Sciex Keto derivatization kit (AB Sciex, Framingham, MA) just prior to LC/MS/MS analysis. To each sample, 50 µL of reagent was added. The reaction time was 60 minutes at room temperature. The samples were transferred to autosampler vials and immediately analyzed.

An Agilent 1200 Rapid Resolution Liquid Chromatography system coupled to an Agilent 6460 series QQQ mass spectrometer was used to analyze all samples after derivatization at the Bindeley Bioscience Center at Purdue University, IN. For the dansyl chloride derivatives a Waters Xbridge C18 2.1 mm x 100 mm, 3-µm column was used for LC separation. The buffers were (A) water + 0.1 % formic acid and (B) acetonitrile + 0.1 % formic acid. The linear LC gradient was as follows: time 0 minutes, 10 % B and 90 % A; time 5 minutes, 100 % B and 0 % A; time 15.5 minutes, 10 % B and 90 % A; time 18 minutes, 10 % B and 90 % A. The flow rates of buffers (A) and (B) were 0.3 mL/min. Multiple reaction monitoring was used for MS analysis. The data were acquired in positive electrospray ionization (ESI) mode by monitoring the following transitions: estradiol (dansyl Cl), *m/z* (atomic mass units) 506.1→171 (30 V), m/*z* 155.8 (40 V); d_5-estradiol (dansyl Cl), *m/z* 511.1→171 (30 V), *m/z* 155.8 (40 V); estriol (dansyl

Cl), *m/z* 522→171 (30 V), 155.8 (40 V). This method can also be used to monitor progesterone in its unlabeled form by following the transition: *m/z* 315.2→109 (15 V), 97 (15 V); d_9-progesterone, *m/z* 324.2→113 (15 V), 100 (15 V) if necessary. ESI interface had a nitrogen gas temperature of 325 °C, nitrogen gas flow rate of 8 L/minute, nebulizer pressure of 45 psi, sheath gas temperature of 250 °C, sheath gas flow rate of 7 L/minute, capillary voltage of 3500 V, and nozzle voltage of 1500 V.

For the keto derivatives, the following conditions were used for LC/MS/MS analysis. An Agilent Zorbax 80Å Extend-C18 4.6 mm x 150 mm, 5-µm column was used with the buffers (A) water + 0.1 % formic acid and (B) acetonitrile + 0.1 % formic acid. The linear LC gradient was as follows: time 0 minutes, 10 % B and 90 % A; time 10 minutes, 100 % B and 0 % A; time 12 minutes, 10 % B and 90 % A; time 15 minutes, 10 % B and 90 % A. The flow rates of buffers (A) and (B) were 0.3 mL/min. Multiple reaction monitoring was used for MS analysis. The data were acquired in positive ESI mode by monitoring the following transitions: testosterone, *m/z* 403.1→344.1 (20 V), 164 (40 V); $^{13}C_3$-testosterone *m/z* 406.1→347.1 (20 V), 167 (40 V); cortisol *m/z* 477.1→418.3 (15 V), 388.2 (35 V); d_4-cortisol *m/z* 481.1→422.3 (15 V), 392.3 (35 V); progesterone *m/z* 429.1→370 (20 V), 126 (30 V); d_9-progesterone *m/z* 438.1→379 (20 V), 132 (30 V). The jet stream ESI interface had a nitrogen gas temperature of 325 °C, nitrogen gas flow rate of 8 L/minute, nebulizer pressure of 45 psi, sheath gas temperature of 250 °C, sheath gas flow rate of 7 L/minute, capillary voltage of 4000 V, and nozzle voltage of 1000 V.

Samples with hormone concentrations below detection limit for LC/MS/MS analysis (< 0.5 ng) were included in statistical analysis by assigning one-half the detection limit for each hormone with a non-detectable signal (Gilbert 1987, Dehn *et al.* 2005). Extraction efficiencies

were determined by comparing known volumes of added ISTDs of each added to blank samples that went through the steroid hormone extraction method with no bone, serum, or blubber sample (n = 8, "Blank-Extraction") to samples with known volumes of added ISTDs that were dried with nitrogen gas and no extraction (n = 5, "Blank-Dried ISTDs"). The percent recovery of each ISTD was calculated by comparing the ratio of mean hormone concentration detected in "Blank-Extraction", divided by the mean hormone concentration in the "Blank-Dried ISTDs" samples. The mean extraction efficiencies for each hormone in walrus bone, blubber, and serum are as follows: progesterone = 51 %, testosterone = 107 %, cortisol = 72 % and estradiol = 79 %. All reported hormone concentrations from all tissues were corrected for extraction efficiency.

2.3.4 Statistical Analysis

Walrus bone samples came from various types of skeletal elements, but mostly from flipper long bones. However, UAM provided skull and mandible bone samples from the same individual walruses to assess potential differences of steroid hormone concentrations between different bone elements. Paired two-tailed t-tests were performed on skull and mandible from the same individual walrus to determine if hormone concentrations were significantly different between bone elements (n = 7 pairs).

Data were log transformed to normalize distribution of steroid hormone concentrations in all tissues. Three factorial Analysis of Variance (ANOVAs) tests were used to test for differences in mean concentrations of steroid hormones among bone, blubber, and serum, between sexes, and the interaction of sex and tissue, with Tukey *post hoc* tests. The majority of animals were classified as adult walruses (n = 25 adults, n = 3 subadults, and n = 5 unknown), therefore, sample size was too small to perform statistical analysis among age classes (Appendix

2.1). Bivariate general linear models were used to determine if any significant predictor correlations could be determined among hormone concentrations of different tissues (Kellar *et al.* 2013). Non-metric Multidimensional Scaling (nMDS) plots based on a Bray-Curtis similarity matrix with 50 restarts were used to visually illustrate similarities among different tissues based on steroid hormone concentrations and between years *(i.e.*, 2014 and 2015). One-way Similarity Percentages Analysis (SIMPER) was used to determine which hormone concentrations contributed to differences between 2014 and 2015 samples (Mejri *et al.* 2014). ANOVAs, paired two-tailed t-tests, general linear models, and graphical representations of data were done in the core programming of R (R Core Team 2013). SIMPER and nMDS analyses were performed in Primer (V6, Clarke and Gorley 2006). Bone, blubber, and serum hormone concentrations were reported as ng/g bone powder, ng/g blubber, and ng/mL serum, respectively (ng/g, ng/g, and ng/mL, respectively hereafter). An alpha of 0.05 was used to determine significant differences and correlations among tissue steroid hormone concentrations. Results are reported as mean +/- 1 standard deviation unless otherwise noted.

2.3.5 Estimated Bone Hormone Turnover Rate

Bone steroid hormones have not been studied in marine mammals (and only very limited data are available for terrestrial mammals), and therefore the turnover of bone hormone concentrations is unknown (Yarrow *et al.* 2010). Lipids, and the steroid hormones attached to them, are present in both cortical and marrow bone tissue (During *et al.* 2015). Lipids in cortical bone either reside in osteocytes, which are embedded in the cortical bone or are directly associated with the cortical bone matrix compounds (During *et al.* 2015). Only cortical bone tissue was sampled in this study. Therefore, the calculated estimate of walrus bone steroid

hormone turnover in this study was based on human cortical bone turnover rate (Clarke 2008), the human cortical bone skeletal makeup (*i.e.*, 75 % of human body is composed of cortical bone, Clarke 2008), and Pacific walrus skeletal biological information (*i.e.*, median weight of female and male walruses and their skeletons, Fay 1982, Table 2.3). There is variation in the adult human cortical bone turnover rate of 2 - 3 % / year (Clarke 2008). However, Clarke (2008) does not give information about sex specific cortical bone turnover rates. Therefore, we chose the highest rate of 3 % / year, because this would give us a minimum reservoir time of steroid hormones in walrus cortical bone. This turnover rate is an estimate, and it is used to argue that bone hormones are mean concentrations representing an individual's lifetime. Further study is needed to confirm this bone time signature for steroid hormone concentrations.

2.4 Results

2.4.1 Steroid Hormone Concentrations in Bone Elements and Non-Detectable Samples

Steroid hormone concentrations were not significantly different between paired mandible and skull bone elements of walruses (paired two-tailed t-test, cortisol $P = 0.32$, estradiol $P = 0.08$, progesterone $P = 0.20$, and testosterone $P = 0.11$, $n = 7$ pairs); this is in agreement with Yarrow *et al.* (2010). Therefore, we were able to compare bone steroid hormone concentrations from the various bone elements that hunters opportunistically collected for this study.

There were a few non-detectable (ND; < 0.5 ng) hormone concentrations. In bone samples, progesterone concentrations were below detection limits in two samples ($n = 2$). In blubber, all 2015 male samples, excluding one duplicate, were below detectable limits for progesterone ($n = 19$, total 2015 male blubber samples $n = 20$). Estradiol in blubber from 2015 was below detectable limits in 20 of 22 samples (including duplicates from the one 2015 female).

There were no samples from any tissue below detectable limits for cortisol or testosterone hormone concentrations. Further, all serum samples had detectable concentrations of all steroid hormones analyzed.

2.4.2 Steroid Hormone Concentrations in Females and Interannual Variability

Tables 2.1 and 2.2 report a summary of mean steroid hormone concentrations and *P* values from the ANOVAs for each tissue analyzed in both male and female walruses. Female reproductive information was collected when hunters harvested them (Table 2.4). Sample size (n = 6 total females sampled) was too small to assess differences in hormone concentrations among female reproductive status (Table 2.4). However, the one female sampled in 2015 had two orders of magnitude lower bone, blubber, and serum estradiol concentrations compared with females collected in 2014 (Table 2.4). The 2015 female had a yearling present when hunted and other steroid hormone concentrations from this 2015 female were comparable to other 2014 females with either a calf or yearling (Table 2.4). The only pregnant female sampled had the highest blubber progesterone concentration (141.98 ng/g) among all females. In addition, the pregnant female had an order of magnitude greater blubber progesterone concentration when compared to the female that was not accompanied by a calf or yearling and was not lactating (21.23 ng/g, Table 2.4). Serum cortisol concentrations from lactating females with calves or yearlings present were higher (60.00 ± 23.83 ng/mL, n = 4) than non-lactating females without a calf or yearling present (28.21± 6.88 ng/mL, n = 2).

Tissues visually grouped together by tissue type based on all steroid hormone concentrations (nMDS, Figure 2.2). However, samples from 2014 and 2015 formed distinctly separate groups based on all steroid hormone concentrations (nMDS, Figure 2.3). The

dissimilarity among samples collected from 2014 compared with samples from 2015 was 85 % (One-Way SIMPER). Differences in estradiol, progesterone, and cortisol concentrations among samples from 2014 and 2015 contributed 74 %, 12 %, and 9 % for estradiol, progesterone, and cortisol, respectively. Differences in testosterone concentrations between years contributed the least (5%) to the dissimilarity in hormone concentrations among 2014 and 2015 samples. To insure this was not due to the higher male sample size (n = 28) compared with females (n = 6), analyses were performed with only males. Dissimilarity remained at 85 % (One-Way SIMPER). Similar to the analysis with both sexes, differences in estradiol, progesterone, and cortisol concentrations among male walruses from 2014 and 2015 contributed 77 %, 10 %, and 7 %, respectively.

2.4.3 Differences in Steroid Hormone Concentrations among Tissues

2.4.3.1 Cortisol

Mean cortisol concentrations (Table 2.1) in all tissues were not different between sexes (ANOVA, P = 0.06), nor was the interaction between sex and tissue significant (P = 0.14), but mean cortisol concentrations were significantly different among tissues (P = < 0.001). Serum cortisol concentrations were significantly higher than walrus blubber and bone (Tukey *post hoc*, P = < 0.001, < 0.001, respectively), but blubber and bone cortisol concentrations were similar (P = 0.96).

2.4.3.2 Estradiol

Due to the high interannual variability contributed by differences in estradiol concentrations between years (SIMPER, 74%, Figure 2.3), concentrations were tested for

significant differences between years. Estradiol concentrations were significantly different between years (ANOVA, $P = < 0.001$, Figure 2.4 A). Thus, 2014 samples were tested for estradiol concentration differences among tissues, between sexes, and the interaction of sex and tissue sampled (Table 2.2). Samples from 2015 only contained one female, thus only differences among tissues were tested (Table 2.2). Samples from 2014 had similar mean estradiol concentrations between sexes with no effect of the interaction of sex and tissue ($P = 0.06$, 0.96, respectively), but concentrations were significantly different among tissues ($P = < 0.001$). Bone and blubber estradiol concentrations were similar (Tukey *post hoc*, $P = 0.51$), but both were significantly higher than serum ($P = < 0.001$, < 0.001, respectively). However, samples from 2015 had similar concentrations among tissues ($P = 0.38$). Overall, mean estradiol concentrations were highly variable between years due to the ND 2015 samples (Table 2.2, Figure 2.4 A). Walrus females had lower estradiol concentrations compared with males from their respective sampling years with the exception of the 2015 female, who had a higher bone estradiol concentration compared with the mean bone concentration from the 2014 males (Table 2.1). Females from 2014 had the highest estradiol concentrations measured in blubber (118.29 ± 6.71 ng/g) and lowest in serum (91.89 ± 6.39 ng/mL) compared with the 2015 female, who had the highest estradiol concentrations measured in bone (1.44 ng/g) and lowest in blubber (0.97 ng/g). Males from 2014 had the highest estradiol concentrations measured in blubber (124.73 ± 16.74 ng/g) and lowest in serum (98.83 ± 25.34 ng/mL) compared with males from 2015, where the highest estradiol was measured in serum (1.83 ± 1.28 ng/mL) and the lowest in blubber (1.21 ± 0.51 ng/g).

2.4.3.3 Progesterone

Mean progesterone concentrations were significantly different between sexes (ANOVA, $P = < 0.001$) and the interaction of sex and tissue ($P = 0.009$), but not tissue as a main effect ($P = 0.27$). Female blubber progesterone concentrations were driving the significant differences seen in the interaction term (*i.e.*, sex*tissue). Female blubber progesterone concentrations were significantly higher compared with male's blubber, serum, and bone progesterone concentrations (Tukey *post hoc*, $P = < 0.001$, 0.007, 0.001, respectively). Similar to estradiol, females had higher mean progesterone concentrations in each tissue sampled (*i.e.*, bone, blubber, and serum) compared with males (Table 2.1). Mean progesterone concentrations were highest overall in blubber of female walruses (85.50 ± 45.44 ng/g) and lowest overall in male blubber (4.47 ± 3.58 ng/g).

2.4.3.4 Testosterone

Mean testosterone concentrations were significantly different among tissues (ANOVA, $P = 0.005$), but not between sexes ($P = 1.0$), nor the interaction of sex and tissue ($P = 0.75$). Significant differences among walrus tissues were only found among bone and blubber testosterone concentrations (Tukey *post hoc*, $P = 0.003$), but not between serum and blubber ($P = 0.33$) or serum and bone ($P = 0.26$). Females had higher mean testosterone concentrations compared with males in blubber (8.26 ± 5.74 ng/g, 8.17 ± 7.01 ng/g, respectively), and bone (16.72 ± 19.62 ng/g, 13.25 ± 11.53 ng/g, respectively), but males had higher mean testosterone concentrations in serum (8.50 ± 3.05 ng/mL, 6.88 ± 2.95 ng/mL, respectively). A summary of mean testosterone concentrations in all tissues is found in Table 2.1.

2.4.4 Correlations among Tissue Steroid Hormone Concentrations

Males and females were pooled if "sex" or the interaction term "sex*tissue" was not significant based on ANOVA. Thus, cortisol, estradiol, and testosterone were pooled (Table 2.5). As stated, progesterone concentrations were significantly different between males and females, and were therefore analyzed separately in the linear regression analysis (Table 2.5). Due to the differences detected in the 2014 and 2015 sampling years, estradiol concentrations in males and females were pooled within their respective sampling year, and correlations among estradiol tissue concentrations were analyzed separately by year. Specific correlations for each hormone concentration among the different tissues are discussed separately below.

2.4.4.1 Cortisol

There were no significant correlations in cortisol concentrations among tissues (Table 2.5). Bone and blubber cortisol concentrations had the weakest correlation (Linear Regression, $P = 0.99$, $R^2 = 7.2 \times 10^{-6}$), while the correlation between bone and serum cortisol was the strongest, but still relatively weak ($P = 0.05$, $R^2 = 0.17$).

2.4.4.2 Estradiol

Estradiol concentrations showed no significant correlations among any tissues from separate collection years, 2014 and 2015 (*i.e.*, $P = > 0.05$ for all comparisons, Table 2.5).

2.4.4.3 Progesterone

Progesterone concentrations in different walrus tissues were analyzed separately for males and females in the linear regression analyses. Blubber and bone progesterone concentrations had a significant correlation in males ($P = < 0.001$, $R^2 = 0.51$). However, bone

and serum progesterone, as well as blubber and serum, were not significant in males ($P = 0.51$, 0.48, respectively). Female bone and blubber progesterone concentrations did have a strong correlation ($R^2 = 0.75$), however it was not significant ($P = 0.06$) likely due to sample size limitations (Table 2.5). There were no significant correlations among any tissues in females (Table 2.5).

2.4.4.4 Testosterone

Testosterone showed no significant correlations among all tissues (Table 2.5). Bone and serum had the weakest correlation (Linear Regression, $P = 0.35$, $R^2 = 0.04$), while the strongest correlation was between blubber and serum ($P = 0.07$, $R^2 = 0.18$).

2.5 Discussion

Our overall goal was to establish how bone steroid hormone concentrations relate to walrus tissues that were collected by hunters and commonly used to monitor stress response of marine mammals, with our study focusing only on blubber (Kellar *et al.* 2015, Vu *et al.* 2015, Kershaw and Hall 2016) and serum (Bartsh *et al.* 1992, Gardiner and Hall 1997, Oki and Atkinson 2004, Harcourt *et al.* 2010, Myers *et al.* 2010). Our study presents the first extraction, validation, and measurement of cortisol, estradiol, progesterone, and testosterone from marine mammal bone. The first main objective of this study was to determine if there were significant differences in reproductive and stress steroid hormone concentrations between different sexes and among various tissues (bone, blubber, and serum) of walruses. In general, we did not find consistent differences in steroid hormones between sexes regardless of tissue type or the interaction of sex and tissue type with the exception of progesterone (Tables 2.1, 2.2). However,

differences were evident for estradiol, cortisol, and testosterone among tissue types (Tables 2.1, 2.2). Our second objective was to determine if any significant correlations among hormone concentrations could be established in different tissues. Progesterone measured in male walrus bone and blubber was the only tissue/hormone correlation that was significant (Table 2.5).

2.5.1 Steroid Hormone Concentrations in Different Tissues and Sexes

2.5.1.1 Cortisol

Cortisol is a biomarker for stress response in mammals, because glucocorticoid hormones (including cortisol) signal various physiological and behavioral changes helping an animal to cope with and adapt to various stressors (Norris 1997, Romero 2004). The release of cortisol in response to an acute stressor helps deliver fatty acids into the bloodstream to aid in the "fight or flight" response, suppress the immune system to prevent an overshooting of a bodily immune response to a pathogen, and mobilize lipid stores for times of fasting (Norris 1997, Sapolsky *et al.* 2000, Romero 2004). However, chronic stressors causing a sustained stress response can be detrimental to an animal by causing a failure to regulate immune responses (Cohen *et al.* 2012), inhibiting reproduction, and can lead to poor body condition through muscle degradation (Norris 1997, Sapolsky *et al.* 2000, Romero 2004).

Serum cortisol concentrations measure the acute stress response of an animal to a stressor and are used regularly in marine mammal studies (reviewed by Amaral 2010). While mean cortisol concentrations in serum were significantly higher than in bone and blubber for both male and female walruses, they were lower than in serum of other pinnipeds, including captive adult Steller sea lions (*Eumetopias jubatus*, Myers *et al.* 2010) and free-ranging adult male Weddell seals (*Leptonychotes weddellii*, Harcourt *et al.* 2010). Serum hormone concentrations represent

circulating (Kellar *et al.* 2013) cortisol concentrations an animal is experiencing when sampled, and may change rapidly due to a number of factors. For example, within 5 minutes of being captured, serum cortisol concentrations of Weddell seals significantly increased from 350 ng/mL to 386 ng/mL (Harcourt *et al.* 2010). Different factors affect naturally circulating cortisol concentrations in different pinnipeds, including capture stress response, age class, diel changes, season (breeding or non-breeding), sex, and location (Bartsh *et al.* 1992, Gardiner and Hall 1997, Oki and Atkinson 2004, Harcourt *et al.* 2010, Myers *et al.* 2010). Therefore, no direct comparisons can or should be made between walruses and other pinniped species. However, a comparison of general patterns is potentially more useful.

Significantly higher serum cortisol concentrations in both sexes compared with blubber and bone may represent an acute stress response to hunting (Harcourt *et al.* 2010), or possibly be characteristic of their natural circulating cortisol concentrations during the post breeding season, as our walruses were obtained in May after the winter breeding season (Fay 1982, Gardiner and Hall 1997). Our results for male walrus serum cortisol concentrations (20.80 ± 7.07 ng/mL) are similar to those from free-ranging male Atlantic walruses when light anesthesia was administered before blood sampling (median = 23.58 ng/mL, Tryland *et al.* 2009), which lowers the acute capture stress effect on serum cortisol concentrations (Harcourt *et al.* 2010). Therefore, having similar serum cortisol concentrations among males from the Pacific and Atlantic populations suggests general circulating cortisol concentrations are representative among males from each walrus subspecies. Females had generally higher mean cortisol serum concentrations than males (Table 2.1), similar to trends observed in other pinnipeds (Gardiner and Hall 1997, Myers *et al.* 2010) and cetaceans (Suzuki *et al.* 1998).

Blubber cortisol concentrations have been measured seasonally in harbor seals, and blubber was argued to be either a reservoir of steroid hormones, including cortisol, or an active endocrine organ producing its own cortisol to mobilize lipids for energy use during times of fasting or high energy activities like molting (Kershaw and Flier 2004, Kershaw and Hall 2016). At this time, blubber is generally accepted as a tissue to monitor longer-term steroid hormone changes in pinnipeds (Kershaw and Hall 2016) and cetaceans (Kellar *et al.* 2013, 2015, Trana *et al.* 2015). However, more research is required to determine if blubber is an active endocrine tissue. Our walrus blubber cortisol concentrations range from 0.66 - 13.17 ng/g, lower than reported for harbor seals (37.84 - 1553.58 ng/g, Kershaw and Hall 2016). In addition, cortisol in blubber has been extracted and measured in male and female walruses (Seymour 2014). Our samples are skewed towards males, while Seymour (2014) included more females; although the male concentration ranges for blubber cortisol were comparable (10.04 - 16.64 ng/g, Seymour, 0.66 - 13.17 ng/g, this study). However, our lower minimum concentrations indicate a higher variability present in male cortisol blubber. While blubber may be a storage reservoir of cortisol (Kershaw and Hall 2016), the time signature that these cortisol concentrations represent in the life of a walrus is unknown. Our samples were collected in May, post breeding season of walruses, because reproductive males and females are rarely seen together after April (Fay 1982). In addition, with walrus spring migrations happening earlier due to early sea ice retreat (Fidel *et al.* 2014), breeding activity may be occurring earlier as well. Therefore, the relatively low blubber cortisol concentrations compared with serum cortisol may not be reflecting increases in blubber cortisol concentrations related to the most recent breeding season (*i.e.*, winter before being harvested).

Ccortisol has not been extracted from walrus or any marine mammal bone until now, but sex steroid hormones have been extracted from rat (Yarrow *et al.* 2010) and human bones (Mark *et al.* 2011). Overall, bone cortisol concentrations were similar to blubber, but significantly lower than serum (Table 2.1). While the mean bone cortisol concentrations were low (Table 2.1), there was one adult male with relatively high concentrations ((M) max = 118.85 ng/g, (F) max = 16.90 ng/g). However, we do not have a tooth estimate for this animal, so its age is unknown. This animal's blubber and serum cortisol concentrations were low compared with bone (3.59 ng/g, 12.75 ng/mL, respectively). Thus, cortisol could have been accumulating in the cortical bone over a longer period of time indicating this animal had been chronically stressed. However, this trend of relatively high bone cortisol compared with blubber and serum was not seen in all samples, as overall mean bone and blubber cortisol concentrations from all samples were similar, and both were lower than serum (Table 2.1). Blubber cortisol concentrations potentially reflect a longer period of accumulation than serum (Kershaw and Hall 2016), which may explain why blubber cortisol concentrations are not significantly different from bone (Table 2.1). This lends support to the idea of bones being a long-term reservoir of steroid hormones, and their utility in monitoring long-term stress response that will not be skewed by acute stressors (Harcourt *et al.* 2010). However, while both serve as a long-term monitor of steroid hormones, bone and blubber have different turnover rates. Our study estimated complete cortical bone turnover in a walrus takes approximately 33 years and applied this turnover rate to steroid hormones in bone (Table 2.3), while blubber has been shown to accumulate steroid hormones on an estimated monthly time period (Trana *et al.* 2015, Kershaw and Hall 2016). Therefore, blubber concentrations at the time walruses were harvested during the spring migration resembled the accumulated lifetime

cortisol signature of the bone, and suggests only limited fluctuations in stress response of walruses over the course of the seasons and years. Both tissues are therefore good tools for long-term monitoring of walrus stress response and can potentially identify chronic stress to individuals and/or the walrus population.

2.5.1.2 Estradiol

In mammals, estradiol prepares the uterus for pregnancy (Norris 1997) and induces lactation (Smith and Schanbacher 1973) and estrus in females (Pomeroy 2011). Estradiol is also important in male reproduction (Hess 2003). Estradiol is synthesized in adult male testicular fluid and maintains fluid reabsorption before sperm enter the epididymis (Hess 2003). A decrease of active estradiol reduces control of fluid reabsorption, ultimately resulting in reduced fertility (Hess 2003). Estradiol has been measured in serum of walruses (Kinoshita *et al.* 2012, Muraco *et al.* 2012, Triggs 2013) and other pinnipeds (Daniel 1974, 1975, Browne *et al.* 2006, Greig *et al.* 2007, Zhang *et al.* 2014).

Mean estradiol concentrations were different between years and among tissues (only 2014 samples), but similar between sexes (Table 2.2). However, concentrations were highly variable across 2014 and 2015 in both males (98.83 ± 25.34 ng/mL, 2014, 1.83 ± 0.96, 2015) and females (91.89 ± 6.39 ng/mL, 2014, 1.34 ng/mL, 2015 female). The high variation seen among females is due to the single lactating female collected in 2015 with very low estradiol serum concentration (Tables 2.2, 2.4). Surges in serum estradiol concentrations in female walruses occur before ovulation (*i.e.*, winter season, Fay 1982), but the duration is short (Kinoshita *et al.* 2012) and would potentially not show up in serum samples collected in May. Walruses contain accessory *corpora lutea* (Fay 1982) which can secrete estradiol whether or not they are pregnant

or pseudopregnant (Kinoshita *et al.* 2012). Perhaps, the higher estradiol concentrations from female walruses in 2014 can be attributed to accessory *corpora lutea* secreting estradiol, while the only female collected in 2015 had not developed any accessory *corpora lutea*. In addition, walruses are capable of delayed implantation and surges of estradiol have been associated with implantation of blastocysts in some pinniped species (Daniel 1974, 1975, Greig *et al.* 2007, Zhang *et al.* 2014). It is possible that the elevation in estradiol concentrations in the 2014 female walruses were due to surges of estradiol relating to the successful implantations that were captured during May, but were not captured with the 2015 female walrus. However, that is unlikely, because implantation is possible in May, but most implantations of blastocysts occur in June and July (Fay 1982).

Male serum estradiol concentrations were also highly variable between years (Table 2.2). Estradiol production in males occurs mostly during spermatogenesis (Hess 2003, Carreau *et al.* 2006), which occurs for walruses primarily from November – March (Fay 1982). Thus, male walruses could have been in early to late stages of spermatogenesis resulting in variable estradiol serum concentrations between years.

Blubber estradiol concentrations were significantly higher in 2014 compared with 2015 walruses (Table 2.2). Blubber estradiol concentrations would potentially remain elevated during estrus, implantation, and spermatogenesis, and for longer periods of time compared with serum if blubber is considered a long-term reservoir of steroid hormones (Kellar *et al.* 2013, Kershaw and Hall 2016). Blubber concentrations were significantly higher than estradiol concentrations measured in serum of 2014 walruses (Table 2.2). Thus, we most likely captured these periods of

estradiol surges in the 2014 samples, but not in the 2015 samples (Table 2.2). Overall, we potentially sampled an array of walruses at various stages of the breeding season.

In humans, adipose tissue is arguably a highly active endocrine organ with the ability to produce androgens, while expressing the enzymes capable of converting androgens to estrogens, including estradiol (Kershaw and Flier 2004). Similar capabilities of local steroid hormone production have been suggested in bowhead whales (Kellar *et al.* 2013) and harbor seals (Kershaw and Hall 2016). However, these authors maintain that circulating steroid hormone concentrations are accumulated in blubber (Kellar *et al.* 2013, Kershaw and Hall 2016). Our results suggest a similar possibility for walrus blubber, as comparable concentrations were measured in blubber and serum in 2015 (Table 2.2). However, further research is warranted into the possible production of estradiol (and other steroid hormones) in walrus blubber.

Estradiol concentrations in walrus bone followed similar trends between years as discussed for serum and blubber (Table 2.2). Based on the bone turnover rate, steroid hormones are potentially accumulated over the lifetime of a walrus and represent an average lifetime steroid hormone time signature (Table 2.3). However, estradiol also stimulates bone turnover, helping to increase bone mineral density, and is locally produced in bone (Yarrow *et al.* 2010, Nguyen *et al.* 2014, During *et al.* 2015). It is still unknown how much this local production of estradiol contributes to overall estradiol concentrations compared with gonadal production, but bone is still an estradiol reservoir to some degree (Yarrow *et al.* 2010). Therefore, our argument of bone being a potential lifetime reservoir of estradiol may be an over estimate of the time estradiol is stored in the bone, and estradiol may not have similar long-term reservoir times compared with other hormones measured in this study. Nevertheless, the similar walrus estradiol

concentrations and relatively same variability between years in bone compared with blubber and serum points to capturing different walruses at various stages of the reproductive season.

2.5.1.3 Progesterone

Progesterone is the main pregnancy hormone that is elevated throughout pregnancy in marine mammals and can be used to determine reproductive status, including pregnancy, in serum of females (Atkinson *et al.* 1999, Zhang *et al.* 2014). Progesterone concentrations were significantly different between sexes with significant differences among tissues only detected when sex was incorporated as an interaction effect (Table 2.1). This means that progesterone concentrations differ between sexes, and are generally lower in males.

Progesterone has been measured in serum of captive walruses (Kinoshita *et al.* 2012, Muraco *et al.* 2012). In agreement with Fay's (1982) observation that the largest *corpus luteum* (*i.e.*, progesterone producing follicle during pregnancy, Pomeroy 2011) in free-ranging walruses occurs throughout March – May, serum progesterone concentrations during this time peaked in captive walruses with maximum serum progesterone concentrations ~30 ng/mL and minimum < 5 ng/mL (Kinoshita *et al.* 2012). Serum progesterone concentrations from our female and male walruses fall within these reported ranges (Table 2.1), and provide the first data for free-ranging walruses (male and female). Serum progesterone concentrations in the 2014 near-term pregnant female walrus were among the lowest measured (4.40 ng/mL). This was also observed in two bowhead whales, where blubber progesterone was high, but serum concentrations were low (blubber/serum ratio > 80, Kellar *et al.* 2013). Kellar *et al.* (2013) suggested the serum progesterone concentrations can drop at a faster rate than blubber based on the one bowhead with reproductive information, a female who had recently given birth. A drop in circulating

progesterone levels has been noted in other mammals as a cue to initiate parturition (Zakar and Hertelendy 2007).

Progesterone measured in blubber has been successfully used to determine reproductive status in cetaceans (Kellar *et al.* 2006, 2013, Trego *et al.* 2013), but not in pinnipeds, including walruses. In this study, the significant difference in the interaction term (*i.e.*, sex*tissue) was driven by the high progesterone concentrations in female blubber (Table 2.1). The 2014 pregnant female had blubber progesterone concentrations that were 7 times higher than the female that was not lactating and had no calf of yearling present (Table 2.4). These ranges are comparable with data reported from immature and pregnant bottlenose dolphins (9 times higher, *Tursiops truncatus*, Pérez *et al.* 2011), but not other cetaceans (164 times higher, pregnant vs. non-pregnant, Trego *et al.* 2013). However, our study requires a larger sample size to relate female walrus reproductive status to progesterone concentrations. Males had lower blubber progesterone concentrations compared with females. This result was not entirely skewed by the pregnant walrus from 2014, as she was removed and a repeat ANOVA determined that significant differences in progesterone concentrations were still present between sexes with differences in tissues being dependent on sampling female blubber (Table 2.1). The lower male blubber progesterone concentrations are expected compared with females (Kellar *et al.* 2013). These are the first reported progesterone concentrations from free-ranging walruses and to our knowledge, for pinnipeds in general.

Bone progesterone concentrations have not been extracted from any marine mammal until this study, but other sex steroid hormones have been extracted from ancient and modern mammal bone (Yarrow *et al.* 2010, Mark *et al.* 2011). Bone progesterone concentrations for

females were lower than blubber, but higher than serum progesterone concentrations (Table 2.1). The females in this study were adults (except for one unknown), and the blubber progesterone is expected to be high due to prolonged circulating progesterone concentrations related to the preceding breeding season (Kellar *et al.* 2013). This is especially true for the pregnant female walrus, which had the highest measured concentrations (141.98 ng/g). However, more unexpectedly, males had significantly higher progesterone concentrations in bone compared with females (Table 2.1).

Progesterone is not only the main female pregnancy hormone, but is also a precursor to other important reproductive (*i.e.*, estradiol and testosterone) and stress (*i.e.*, cortisol) steroid hormones (Koal *et al.* 2012). As mentioned, we argue that bone steroid hormone concentrations are an accumulated average over the lifetime of a walrus (Table 2.3). Males potentially could use cortical bone as a reservoir for progesterone to be metabolized by the active metabolic bone marrow into other important hormones when needed (Yarrow *et al.* 2010, During *et al.* 2015). For example, in rats, stress can reduce circulating testosterone concentrations, but when injected with biologically high progesterone concentrations, male reproductive behavior occurred despite low circulating testosterone (Wagner 2006).

2.5.1.4 Testosterone

Testosterone is a sex hormone mostly associated with sexual maturity in males and the production of sperm (Norris 1997). Testosterone concentrations were not significantly different between sexes, but differed among tissues (Table 2.1). Serum testosterone concentrations were similar to blubber and bone, but blubber and bone were significantly different (Table 2.1). A

captive male walrus displayed similar patterns in serum testosterone concentrations related to breeding behavior as seen in other free-ranging pinnipeds (Muraco *et al.* 2012). Our male walrus serum concentrations (4.79 - 14.79 ng/mL) fall within the ranges previously measured in captive walruses (< 2 - 13 ng/mL, Muraco *et al.* 2012). Female serum testosterone concentrations have not been previously measured in walruses, but in this study were lower than males (Table 2.1). In captive spotted seals (*Phoca largha*), female serum testosterone concentrations were below detectable limits (Zhang *et al.* 2014). However, testosterone measured in human serum throughout the female menstrual cycle resulted in significantly higher testosterone during the follicular phase before dropping in later portions of the menstrual cycle (Judd and Yen 1973). The one female from 2015 had the lowest serum testosterone compared with the relatively higher serum testosterone from the 2014 females (Table 2.4). Possibly, females from different years were at different stages of the breeding season. Our testosterone mean extraction efficiency was 107%, indicating cross reactivity of other metabolites when measuring testosterone (Yarrow *et al.* 2010). This could lead to testosterone concentrations, specifically female serum, being over estimnated. However, LC/MS/MS combined with a steroid hormone extraction method would lead to low cross reactivity in testosterone and other hormones in general due to the high sensitivity of the method to differentiate between analysis of targeted steroid hormones and their metabolites (Makin *et al.* 2010). Therefore, cross reactivity may not be a significant factor in high female serum testosterone concentrations.

Blubber testosterone has only been measured in cetaceans (Kellar *et al.* 2009, Vu *et al.* 2015) and displays seasonal increases and decreases related to mating activities, similar to serum testosterone concentrations of pinnipeds (Atkinson and Gilmartin 1992, Zhang *et al.* 2014).

Walruses sampled in this study were undergoing their annual spring migrations after the winter breeding season during the month of May (Appendix 2.1, Fay 1982). Thus, their blubber and serum testosterone concentrations would be low post breeding season (Atkinson and Gilmartin 1992, Kellar *et al.* 2009, Zhang *et al.* 2014).

Mean bone testosterone concentrations among walruses were higher than serum and blubber (Table 2.1). In this study, adult walruses are the only well represented age class, and higher testosterone concentrations in adult male bone could reflect the accumulated average of numerous breeding seasons throughout their lives (Tables 2.1, 2.3). Females had higher bone testosterone concentrations than males (Table 2.1). In females, elevated fecal testosterone concentrations have been associated with pregnancy and dominance behavior in wild hybrid baboons (*Papio* sp., Beehner *et al.* 2005). In human females, elevated saliva testosterone concentrations during the estrus cycle correlated to an increase in attractiveness to males (Welling *et al.* 2007). Elevated testosterone concentrations in plasma of female rats and serum of female humans have been documented during gestation (Judd and Yen 1973, Weisz and Ward 1980). All females in this study, except one, were either accompanied by a calf and/or yearling, lactating, or pregnant, indicating that they were sexually mature (Table 2.4). Similar to males, females in this study were adults, and higher testosterone in bone compared with serum and blubber could indicate older dominant reproductive females. Testosterone is also known to be an important hormone for conversion into estradiol, which helps stimulate bone turnover in humans (Nguyen *et al.* 2014); however, this was not clearly demonstrated in rats (Yarrow *et al.* 2010). Further research is needed to determine how conversion of testosterone into estradiol affects bone turnover rate in walrus bone and the overall testosterone concentrations in walruses.

2.5.2 Correlations among Tissues

There were no significant correlations among tissues and cortisol, estradiol, and testosterone concentrations (Table 2.5). Thus, bone cortisol, estradiol, and testosterone concentrations can not be estimated by serum and blubber steroid hormone concentrations collected from the same individual. This is in agreement with Yarrow *et al.* (2010), where bone estradiol and serum estradiol concentrations had no significant correlations in rats.

Steroid hormones are not physiologically analogous among tissues and are only recently being used as a biological marker for reproductive status and stress response in marine mammals. Cortisol is potentially produced in pinniped blubber (Kershaw and Hall 2016), which could affect correlations among tissues that circulate or store cortisol, but do not produce them. Estradiol has been measured in marine mammal serum (Kinoshita *et al.* 2012, Zhang *et al.* 2014), but never analyzed in marine mammal blubber or bone. Thus, these are the first data related to correlations between estradiol concentrations among bone, blubber, and serum in free-ranging walruses. The lack of a correlation between estradiol concentrations in bone and serum is in agreement with results from rats (Yarrow *et al.* 2010). However, estradiol is locally produced in bone and is also important for bone mineral density (Yarrow *et al.* 2010, Nguyen *et al.* 2014, During *et al.* 2015), which could weaken correlations with serum and blubber. Testosterone in blubber fluctuates seasonally, but is also influenced by stress hormones (*i.e.*, cortisol, Hunt *et al.* 2006, Kellar *et al.* 2009) potentially having confounding effects when creating correlations among testosterone concentrations in other tissues. Testosterone is also a precursor to estradiol in bone (Yarrow *et al.* 2010, Nguyen *et al.* 2014), which could influence

correlations of serum and blubber with bone. Our results did contrast Yarrow *et al.* (2010), where testosterone bone concentrations were correlated with serum testosterone concentrations in rats.

Tissue comparisons among hormones in marine mammals are rare and, to our knowledge, only exist for bowhead whales (Kellar *et al.* 2013). These authors compared progesterone concentrations among urine, blubber, and serum and determined significant positive correlations of serum and blubber progesterone concentrations, when pregnant animals were included in the analysis. The correlation was not significant when pregnant animals were excluded (Kellar *et al.* 2013). In our study, walrus progesterone concentrations were significantly different among tissues, but only when sampling female blubber due to high concentrations (Table 2.1). Our results contrast Kellar *at al.* (2013) with serum and blubber progesterone from both sexes showing no significant correlation (Table 2.5). This could be due to three factors. First, our sample size was smaller for females in general, and included only one near-term pregnant female. Pregnancy drove the correlation in bowhead whale progesterone between serum and blubber (Kellar *et al.* 2013). Second, we did not pool males and females in the linear regression analysis due to a significant interaction term of sex and tissue, which was only found for progesterone (Table 2.1). Third, the age classes represented in our dataset are skewed towards adults compared with Kellar *et al.* (2013), who sampled mainly younger animals. Our study therefore does not include immature animals, which most likely would have lower progesterone concentrations compared with the relatively higher progesterone concentrations found in adults (Gardiner and Hall 1997, Hunt *et al.* 2014, Zhang *et al.* 2014). However, a strong positive correlation was found for males in this study between progesterone in blubber and bone (Table 2.5). While the correlation for females was not significant, the linear regression coefficient was

strong ($R^2 = 0.75$), another indication that small sample size affected outcomes for females. Therefore, progesterone measured in blubber could estimate bone progesterone concentrations. This will allow walrus researchers to compare progesterone blubber concentrations sampled from the free-ranging, present-day walrus population (*e.g.*, via biopsy) with progesterone concentrations measured in bones collected from archaeological and historical time periods.

Overall, a greater sample size and inclusion of different age classes (*i.e.*, juveniles and sexually immature animals) will likely help improve these findings. Collection of various tissue samples from individual, free-ranging walruses is a unique and rare opportunity, and our data give insight into how steroid hormone concentrations relate among adult male and female walruses.

2.5.3 Differences in Estradiol Between 2014 and 2015 Samples

Significant differences in estradiol concentrations among all walrus samples (males and females) and tissues analyzed from 2014 and 2015 explained 74% of the variation among samples collected from different years (Figure 2.3, Table 2.2). This could be due to a strong bias towards males sampled during 2015. However, males from 2014 had higher estradiol concentrations in all tissues compared with males from 2015 (Table 2.2). This finding of overall low estradiol concentrations in males from 2015, including numerous non-detectable estradiol concentrations, is not abnormal (Zhang *et al.* 2014). However, estradiol concentrations in all tissues of males from 2014 were high and similar to females in 2014 (Table 2.2). Estradiol does not remain elevated for extended periods of time in walruses compared with progesterone, which may stay elevated for up to nine months throughout a pregnancy (Kinoshita *et al.* 2012, Muraco *et al.* 2012, Triggs 2013). Surges of estradiol (as previously mentioned) are more difficult to

capture compared with progesterone. Thus, these differences in estradiol concentrations between years could indicate changes in the timing of the breeding season.

The seasonal migrations of female walruses from their winter breeding grounds in the northern Bering Sea to their summer feeding grounds in the Chukchi Sea depend on the timing of formation and melting of sea ice in the Arctic (Fay 1982, MacCracken 2012, Huntington *et al.* 2015). The winter sea ice melt date for 2014 started on March 21, 2014 (NSIDC 2014). Walruses sampled from 2014 would have sufficient winter sea ice serving as a reproductive platform and could stay later to breed (Fay 1982) compared with walruses harvested in 2015. In 2015, winter sea ice began melting earlier than average and also reached the lowest recorded satellite winter extent on February 25, 2015 (NSIDC 2015). Walruses from both years were harvested at generally the same time of year (May 2014/2015, Appendix 2.1), reducing variability in estradiol concentrations due to harvesting date between years. Most breeding behavior of males (*e.g.*, whistles and clicks) are observed in March (Fay 1982), and thus fertilization mostly occurs during that time based on the timing of the implantation of the blastocysts in June and July (delayed implantation lasts 4 – 5 months in walrus, Fay 1982). Therefore, the significantly lower estradiol concentrations in male and female walrus tissues from 2015 compared with 2014 walruses could either be due to a shortened active breeding season due to lack of available sea ice in March (Fay 1982, Table 2.2), or simply, they did not breed that year. An increase in ice free water (5.5 months to 8.5 months) in the Bering Sea, and different timing of the passing of sea ice through the Bering Strait is expected to occur by the end of the century (Douglas 2010). Most likely, walruses will have to adapt their breeding activity to these changes.

The significantly different concentrations of estradiol between walrus bone samples collected in 2014 and 2015 (Table 2.2, Figure 2.4 A) indicates that estradiol is locally produced in walrus bone, and its reservoir in cortical bone is more readily modifiable compared with other bone steroid hormones measured in this study (Figure 2.4 B, Yarrow *et al.* 2010). However, bone is still a longer-term reservoir of estradiol compared with serum, because estradiol measured in bone is not significantly affected by acute changes in serum estradiol concentrations (Yarrow *et al.* 2010). Thus, cortical bone may be accumulating estradiol on a yearly timescale, compared with the estimated accumulated lifetime average of 33 years represented by the other hormone concentrations in this study (Table 2.3). This potential difference in reservoir time must be taken into account when interpreting estradiol concentrations measured in bone and using estradiol as a long-term monitor of walrus reproductive status.

2.5.4 Conclusions

We were able to quantify cortisol, estradiol, progesterone, and testosterone in bone, blubber, and serum from individual, free-ranging walruses and analyzed correlations of steroid hormone concentrations among tissues. We focused on bone, because blubber and serum are not available from archaeological and historical time periods, making bone an essential tissue for monitoring long-term changes in stress response and reproductive status of the walrus via steroid hormone studies in response to warming in the Arctic. Our results support walrus cortical bone as an estimated accumulated lifetime average reservoir of cortisol, progesterone, and testosterone. However, estradiol in cortical bone had high interannual variability and most likely represents a yearly hormone accumulation, which should be considered when interpreting estradiol bone concentrations. Blubber and serum cortisol concentrations cannot estimate bone

cortisol concentrations and *vice versa*. Thus, bone cortisol concentrations should be analyzed for future monitoring of the stress response of walruses, if researchers wish to compare the stress response of modern walruses to archaeological and historical walruses stress response determined from bone. We showed a significant positive correlation among bone and blubber progesterone concentrations. Thus, progesterone concentrations measured in bone from archaeological and historical time periods can be compared with blubber progesterone concentrations collected from ongoing walrus biopsy efforts. This allows the continuation of long-term monitoring of the reproductive status of the walrus population throughout the current warming in the Arctic. However, further research including collection of different age classes, different female reproductive status, and different seasons, could present better correlations of steroid hormones among different walrus tissues.

2.6 Acknowledgements

The authors thank the Native subsistence hunters from the St. Lawrence Island communities of Savoonga and Gambell, who provided bone, blubber, and serum of their walruses during 2014 and 2015. Without their dedication and support this project could not have been done. We gratefully acknowledge our collaborators: the University of Alaska Museum, U.S. Fish and Wildlife Service, Eskimo Walrus Commission, and the Alaska Department of Fish and Game. A special thank-you to Dr. M. Wooller for his helpful review and comments of this manuscript. We specifically thank C. Clark and the undergraduate laboratory technicians, S. George, C. Markel, L. Wendling, E. VanDam and many others, who provided assistance with sample preparation and tissue extractions throughout this project. This work was funded by the National Science Foundation Arctic SEES Program, Grant No. 1263848, with partial

contributions by the Bureau of Ocean Energy Management. Additional funding was provided by Center for Global Change at the University of Alaska Fairbanks.

2.7 References

Accorsi, P. A., E. Carloni, P. Valesecchi, R. Viggiani, M. Gameroni, C. Tamanini and E. Seren. 2008. Cortisol determination in hair and faeces from domestic cats and dogs. General and Comparative Endocrinology 155:398–402.

Amaral, R. 2010. Use of alternative matrices to monitor steroid hormones in aquatic mammals: A review. Aquatic Mammals 36:162–171.

Atkinson, S., and W. G. Gilmartin. 1992. Seasonal testosterone pattern in Hawaiian monk seals (*Monachus schauinslandi*). Journal of Reproduction and Fertility 96:35–39.

Atkinson, S., C. Combelles, D. Vincent, P. Nachtigall, J. Pawloski and M. Breese. 1999. Monitoring of progesterone in captive female false killer whales, *Pseudorca crassidens*. General and Comparative Endocrinology. 115:323–332.

Bartsh, S. S., S. D. Johnston and D. B. Siniff. 1992. Territorial behavior and breeding frequency of male Weddell seals (*Leptonychotes weddelli*) in relation to age, size, and concentrations of serum testosterone and cortisol. Canadian Journal of Zoology 70:680–692.

Beehner, J. C., J. E. Phillips-Conroy and P. L. Whitten. 2005. Female testosterone, dominance rank, and aggression in an Ethiopian population of hybrid baboons. American Journal of Primatology 67:101–119.

Bluhm, B., and R. Gradinger. 2008. Regional variability in food availability for Arctic marine mammals. Ecological Applications 18:77–96.

Browne, P., A. J. Conley, T. Spraker, R. R. Ream and B. L. Lasley. 2006. Sex steroid concentrations and localization of steroidogenic enzyme expression in free-ranging female northern fur seals (*Callorhinus ursinus*). General and Comparative Endocrinology 147:175–183.

Bryan, H. M., A. G. Adams, R. M. Invik, K. E. Wynne-Edwards and J. E. G. Smits. 2013. Hair as a meaningful measure of baseline cortisol levels over time in dogs. Journal of the American Association for Laboratory Animal Science 52:189–96.

Burek, K. A., F. M. D. Gulland and T. M. O'Hara. 2008. Effects of climate change on Arctic marine mammal health. Ecological Applications 18:126–134.

Carreau, S., C. Delalande, D. Silandre, S. Bourguiba and S. Lambard. 2006. Aromatase and estrogen receptors in male reproduction. Molecular and Cellular Endocrinology 246:65–68.

Carroll, S. S., L. Horstmann-Dehn and B. L. Norcross. 2013. Diet history of ice seals using stable isotope ratios in claw growth bands. Canadian Journal of Zoology 91:191–202.

Clarke, K. R., and Gorley R. N. 2006. PRIMER v6: User manual/tutorial. PRIMER-E, Plymouth, UK 192pp.

Clarke, B. 2008. Normal bone anatomy and physiology. Clinical Journal of the American Society of Nephrology 3:131–139.

Cohen, S., D. Janicki-Deverts, W. J. Doyle, G. E. Miller, E. Frank, B. S. Rabin and R. B. Turner. 2012. Chronic stress, glucocorticoid receptor resistance, inflammation, and disease risk. Proceedings of the National Academy of Sciences of the United States of America 109:5995–9.

Cooper, L.W., C. J. Ashjian, S. L. Smith, L. A. Codispoti, J. M. Grebmeier, R. G. Campbell and E.B. Sherr. 2006. Rapid seasonal sea-ice retreat in the Arctic could be affecting Pacific walrus (*Odobenus rosmarus divergens*) recruitment. Aquatic Mammals 32:98–102.

Daniel, J. C. 1974. Circulating levels of oestradiol-17β during early pregancy in the Alaskan fur seal showing an oestrogen surge preceding implantation. Journal of Reproduction and Fertility 37:1969–1972.

Daniel, J. C. 1975. Concentrations of circulating progesterone during early pregnancy in the Northern ful seal, (*Callorhinus ursinus*). Journal of the Fisheries Research Board of Canada 32:65–66.

Dehn, L. A., G. G. Sheffield, E. H. Follmann, L. K. Duffy, D. L. Thomas, G. R. Bratton, R. J. Taylor and T. M. O'Hara TM. 2005. Trace elements in tissues of phocid seals harvested in the Alaskan and Canadian Arctic: influence of age and feeding ecology. Canadian Journal of Zoology 83:726–746.

Difrancesco, R., V. Frerichs, J. Donnelly, C. Hagler, J. Hochreiter and K. Tornatore. 2007. Simultaneous determination of cortisol, dexamethasone, methylprednisolone, prednisone, prednisolone, mycophenolic acid and mycophenolic acid glucuronide in human plasma utilizing liquid chromatography-tandem mass spectrometry. Journal of Chromatography B 859:42–51.

Douglas, D. C. 2010. Arctic sea ice decline: Projected changes in timing and extent of sea ice in the Bering and Chukchi Seas: U.S. Geological Survey Open-File Report 2010-1176. U.S. Geological Survey, Reston VA.

During, A., G. Penel and P. Hardouin. 2015. Understanding the local actions of lipids in bone physiology. Progress in Lipid Research 59:126–146.

Evershed, R. P., G. Turner-Walker, R. E. M. Hedges, N. Tuross and A. Leyden. 1995. Preliminary results for the analysis of lipids in ancient bone. Journal of Archaeological Science 22:277–290.

Fair, P. A., and P. R. Becker. 2000. Review of stress in marine mammals. Journal of Aquatic Ecosystem Stress and Recovery 7:335–354.

Fay, F. H. 1982. Ecology and biology of the Pacific walrus, *Odobenus Rosmarus Divergens* Illiger. North American Fauna 74:1–266.

Fay, F. H., and J. J. Burns. 1988. Maximal feeding depth of walruses. Arctic 41:239–240.

Fay, F. H., L. L. Eberhardt, B. P. Kelly, J. J. Burns and L. T. Quakenbush. 1997. Status of the Pacific walrus population, 1950-1989. Marine Mammal Science 13:537–565.

Fidel, M., A. Kliskey, L. Alessa and O. P. Sutton. 2014. Walrus harvest locations reflect adaptation: a contribution from a community-based observation network in the Bering Sea. Polar Geography 37:48–68.

Gardiner, K. J., and A. J. Hall. 1997. Diel and annual variation in plasma cortisol concentrations among wild and captive harbor seals (*Phoca vitulina*). Canadian Journal of Zoology 75:1773–1780.

Garlich-Miller, J. L., L. T. Quakenbush and J. F. Bromaghin. 2006. Trends in age structure and productivity of Pacific walruses harvested in the Bering Strait region of Alaska, 1952-2002. Marine Mammal Science 22:880–896.

Gilbert, R. O. 1987. Statistical methods for environmental pollution monitoring. John Wiley and Sons, Inc., New York.

Grebmeier, J. M. 2012. Shifting patterns of life in the pacific Arctic and sub-Arctic Seas. Annual Review of Marine Science 4:63–78.

Greig, D. J., K. L. Mashburn, M. Rutishauser, F. M. D. Gulland, T. M. Williams and S. Atkinson. 2007. Seasonal Changes in Circulating Progesterone and estrogen concentrations in the California sea lion (*Zalophus Californianus*). Journal of Mammalogy 88:67–72.

Gulland, F. M. D., M. Haulena and B. Hilliard. 1999. Adrenal function in wild and rehabilitated Pacific harbor seals (*Phoca vitulina richardsii*) and in seals with phocine herpesvirus-associated adrenal necrosis. Marine Mammal Science 15:810–817.

Harcourt, R. G., E. Turner, A. Hall, J. R. Waas and M. Hindell. 2010. Effects of capture stress on free-ranging, reproductively active male Weddell seals. Journal of Comparative Physiology A 196:147–154.

Hess, R. A. 2003. Estrogen in the adult male reproductive tract: A review. Reproductive Biology and Endocrinology 1:52.

Hogg, C. J., T. L. Rogers, A. Shorter, K. Barton, P. J. O. Miller and D. Nowacek. 2009. Determination of steroid hormones in whale blow: It is possible. Marine Mammal Science 25:605–618.

Hunt, K. E., R. M. Rolland, S. D. Kraus and S. K. Wasser. 2006. Analysis of fecal glucocorticoids in the North Atlantic right whale (*Eubalaena glacialis*). General and Comparative Endocrinology 148:260–272.

Hunt, K. E., R. Stimmelmayr, C. George, C. Hanns, R. Suydam, H. Brower Jr. and R. M. Rolland. 2014. Baleen hormones: A novel tool for retrospective assessment of stress and reproduction in bowhead whales (*Balaena mysticetus*). Conservation Physiology 2:1–12.

Huntington, H. P., M. Nelson, and L. T. Quakenbush. 2015. Traditional knowledge regarding walrus, ringed seals, and bearded seals near Barrow, Alaska. Final report to the Eskimo Walrus Commission, the Ice Seal Committee, and the Bureau of Ocean Energy Management for contract #M13PC00015. 8pp.

IPCC. 2013. Summary for policymakers. In: Climate Change 2013: The physical science basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Stocker TF, Qin D, Plattner GK, Tignor M, Allen SK, Boschung J, Nauels K, Xia Y, Bex V, Midgley PM (eds.)] Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Jay, C. V., and A. S. Fischbach. 2008. Pacific walrus response to Arctic Sea ice losses. U.S. Geological Survey, Fact Sheet 2008–3041. 4 pp.

Jay, C. V., A. S. Fischbach and A. A. Kochnev. 2012. Walrus areas of use in the Chukchi Sea during sparse sea ice cover. Marine Ecology Progress Series 468:1–13.

Jay, C. V., J. M. Grebmeier, A. S. Fischbach, T. L. McDonald, L. W. Cooper and F. Hornsby. 2014. Pacific walrus (*Odobenus rosmarus divergens*) resource selection in the Northern Bering Sea. PloS one 9:e93035.

Judd, H., and S. S. Yen. 1973. Serum androstenedione and testosterone levels during the menstrual cycle. The Journal of Clinical Endocrinology & Metabolism 36:475–481.

Kaufman, D. S., D. P. Schneider, N. P. McKay *et al.* 2009. Recent warming reverses long-term Arctic cooling. Science 325:4–7.

Kellar, N. M., M. L. Trego, C. I. Marks and A. E. Dizon. 2006. Determining pregnancy from blubber in three species of delphinids. Marine Mammal Science 22:1–16.

Kellar, N. M., M. L. Trego, C. I. Marks, S. J. Chivers, K. Danil and F. I. Archer. 2009. Blubber testosterone: A potential marker of male reproductive status in short-beaked common dolphins. Marine Mammal Science 25:507–522.

Kellar, N. M., J. Keliher, M. L. Trego, K. N. Catelani, C. Hanns, J. C. George and C. Rosa. 2013. Variation of bowhead whale progesterone concentrations across demographic groups and sample matrices. Endangered Species Research 22:61–72.

Kellar, N. M., K. N. Catelani, M. N. Robbins, M. L. Trego, Allen C. D., K. Danil and S. J. Chivers. 2015. Blubber cortisol: A potential tool for assessing stress response in free-ranging dolphins without effects due to sampling. PLoS ONE 10:1–16.

Kershaw, E., and J. S. Flier. 2004. Adipose tissue as an endocrine organ. Cellular Endocrinology in Health and Disease 89:229–237.

Kershaw, J. L., and A. J. Hall. 2016. Seasonal variation in harbour seal (*Phoca vitulina*) blubber cortisol - A novel indicator of physiological state? Scientific Reports 6:21889.

Kinoshita, K., M. Kiwata, R. Kuwano, *et al.* 2012. Temporal association of serum progesterone concentrations and vaginal cytology in walruses (*Odobenus rosmarus*). Theriogenology 77:933–939.

Koal, T., D. Schmiederer, H. Pham-Tuan, C. Röhring and M. Rauh. 2012. Standardized LC-MS/MS based steroid hormone profile-analysis. Journal of Steroid Biochemistry and Molecular Biology 129:129–138.

MacCracken, J. G. 2012. Pacific walrus and climate change: observations and predictions. Ecology and Evolution. 2:2072–2090.

Makin, H. L .J., J. W. Honour, C. H. L. Shackleton and W. J. Griffiths. 2010. General methods for the extraction, purification, and measurement of steroids by chromatography and mass spectrometry. Pages 163–282 *in* J.H.L. Makin and D.B. Gower, eds. Steroid Analysis, 2nd ed. Springer, Netherlands, Dordrecht.

Mansour, A. A. H., D. W. McKay, J. Lien, J. C. Orr, N. Øien and G. Stenson. 2002. Determination of pregnancy status from blubber samples in minke whales (*Balaenoptera acutorostrata*). Marine Mammal Science 18:112–120.

Mark, L., Z. Patonai, A.Vaczy, N. Kajsza and A. Marcsik. 2011. Hormone mass fingerprinting: Novel molecular sex determination of ancient human skeletal remains. Pages 411–415 *in* I. Turbanti-Memmi, editor. Proceedings of the 37th International Symposium on Archaeometry, 13th-16th May 2008, Siena, Italy. Springer, Berlin, Heidelberg.

Mejri, S., C. Audet, G. W. Vandenberg, C. C. Parrish and R. Tremblay. 2014. Biochemical egg quality in a captive walleye (*Sander vitreus*) broodstock population relative to ovulation timing following hormonal treatment. Aquaculture 431:99–106.

Metcalf, V., and M. Robards. 2008. Sustaining a healthy human-walrus relationship in a dynamic environment: challenges for comanagement. Ecological Applications 18:148–156.

Muraco, H. S., L. D. Coombs, D. G. Procter, P. J. Turek and M. J. Muraco. 2012. Use of human chorionic gonadotropin in a male Pacific walrus (*Odobenus rosmarus divergens*) to induce rut and achieve a pregnancy in a nulliparous female. Journal of Andrology 33:789–97.

Murtagh, R., V. Behringer and T. Deschner. 2013. LC-MS as a method for non-invasive measurement of steroid hormones and their metabolites in urine and faeces of animals. Veterinary Medicine Austria 100:247–254.

Myers, M. J., B. Litz and S. Atkinson. 2010. The effects of age, sex, season and geographic region on circulating serum cortisol concentrations in threatened and endangered Steller sea lions (*Eumetopias jubatus*). General and Comparative Endocrinology 165:72–77.

NSIDC (National Snow and Ice Data Center). 2014. 2014 melt season in review. Posted by J. Beitler. National Snow and Ice Data Center, Boulder, CO. Available at http://nsidc.org/arcticseaicenews/2014/10/2014-melt-season-in-review/ (accessed April 10, 2016).

NSIDC (National Snow and Ice Data Center). 2015. 2015 melt season in review. Posted by J. Beitler. National Snow and Ice Data Center, Boulder, CO. Available at http://nsidc.org/arcticseaicenews/2015/10/2015-melt-season-in-review/ (accessed April 10, 2016).

Nguyen, H. T. T., B.V. Schoultz, T.V. Nguyen, T.X. Thang, T.T. Chau, P.T.M. Duc and A.L. Hirschberg. 2014. Sex hormone levels as determinants of bone mineral density and osteoporosis in Vietnamese women and men. Journal of Bone and Mineral Metabolism 33:658–665.

Norris, D. 1997. Vertebrate Endocrinology, 3rd ed. Academic Press, San Diego, California.

Oki, C., and S. Atkinson. 2004. Diurnal patterns of cortisol and thyroid hormones in the harbor seal (*Phoca vitulina*) during summer and winter seasons. General and Comparative Endocrinology 136:289–97.

Pérez, S., Á. García-López, R. De Stephanis, *et al.* 2011. Use of blubber levels of progesterone to determine pregnancy in free-ranging live cetaceans. Marine Biology 158:1677–1680.

Pomeroy, P. 2011. Reproductive cycles of marine mammals. Animal Reproduction Science 124:184–193.

R Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.URL http://www.R-project.org/.

Romero, L. M. 2004. Physiological stress in ecology: Lessons from biomedical research. Trends in Ecology and Evolution 19:249–255.

Sapolsky, R., L. Romero and A. Munck. 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine Reviews 21:55–89.

Seymour, J. 2014. Pacific walrus use of higher trophic level prey and the relation to sea ice extent, body condition, and trichinellosis. Ph.D. dissertation. University of Alaska, Fairbanks, AK. 293 pp.

Smith, L. K., and F. L. Schanbacher. 1973. Hormone induced lactation in the Bovine. I. Lactational performance following injections of 17β-estradiol and progesterone. Journal of Dairy Science 56:738–743.

Suzuki, M., T. Tobayama, E. Katsumata, M. Yoshioka and K. Aida. 1998. Serum cortisol levels in captive killer whale and bottlenose dolphin. Fisheries Science 64:643–647.

Trana, M. R., J. D. Roth, G. T. Tomy, W. G. Anderson and S. H. Ferguson. 2015. Increased blubber cortisol in ice-entrapped beluga whales (*Delphinapterus leucas*). Journal of Experimental Marine Biology and Ecology 462:8–13.

Trego, M. L., N. M. Kellar and K. Danil. 2013. Validation of blubber progesterone concentrations for pregnancy determination in three dolphin species and a porpoise. PLoS One 8:e69709.

Triggs, L. 2013. Establishing endocrine and behavioral parameters of reproduction in Pacific walrus. M.S. thesis. University of Washington, Seattle, WA. 239 pp.

Trumble, S. J., E. M. Robinson, M. Berman-Kowalewski, C. W. Potter and S. Usenko. 2013. Blue whale earplug reveals lifetime contaminant exposure and hormone profiles. Proceedings of the National Academy of Sciences of the United States of America 110:16922–6.

Tryland, M., C. Lydersen, K. M. Kovacs and S. I. Thoresen. 2009. Serum chemistry reference values in free-ranging North Atlantic male walruses (*Odobenus rosmarus rosmarus*) from the Svalbard archipelago. Veterinary Clinical Pathology 38:501–506.

Udevitz, M. S., R. L. Taylor, J. L. Garlich-Miller, L. T. Quakenbush and J. A. Snyder. 2013. Potential population-level effects of increased haulout-related mortality of Pacific walrus calves. Polar Biology 36:291–298.

USFWS (U.S. Fish and Wildlife Service). 2014. Pacific walrus: The Endangered Species Act and Alaska Native Hunting. ESA Fact Sheet, Anchorage, AK, 1 pp. Retrieved March 11 2016 from: http://www.fws.gov/alaska/fisheries/mmm/walrus/esa.htm

Vu, E. T., C. Clark, K. Catelani, N. M. Kellar and J. Calambokidis. 2015. Seasonal blubber testosterone concentrations of male humpback whales (*Megaptera novaeangliae*). Marine Mammal Science 31:1258–1264.

Wagner, C. K. 2006. The many faces of progesterone: A role in adult and developing male brain. Frontiers in Neuroendocrinology 27:340–359.

Weisz, J., and I. L. Ward. 1980. Plasma testosterone and progesterone titers of pregnant rats, their male and female fetuses, and neonatal offspring. Endocrinology 106:306–316.

Welling, L. L. M., B. C. Jones, L. M. DeBruine *et al.* 2007. Raised salivary testosterone in women is associated with increased attraction to masculine faces. Hormones and Behavior 52:156–161.

Yarrow, J. F., C. F. Conover, J. A. Lipinska, C. A. Santillana, T. J. Wronski and S. E. Borst. 2010. Methods to quantify sex steroid hormones in bone: applications to the study of androgen ablation and administration. American Journal of Physiology - Endocrinology and Metabolism. 299:E841–E847.

Zakar, T., and F. Hertelendy. 2007. Progesterone withdrawal: key to parturition. American Journal of Obstetrics and Gynecology 196:289–296.

Zhang, F., M. J. Bartels, D. R. Geter, M. S. Carr, L. E. McClymount, T. A. Marino, and G. M. Klecka. 2009. Simultaneous quantitation of testosterone, estradiol, ethinyl estradiol, and 11-ketotestosterone in fathead minnow fish plasma by liquid chromatography/positive atmospheric pressure photoionization tandem mass spectrometry. Rapid Communications in Mass Spectrometry 23:3637–3646.

Zhang, P., Y. Yang, J. Han, Z. Lu, L. Wang, J. Tian, Q. Wang. 2014. Serum testosterone, progesterone, and estradiol concentrations and sexual maturation in spotted seals (*Phoca largha*). Theriogenology 82:475–480.

2.8 Figures

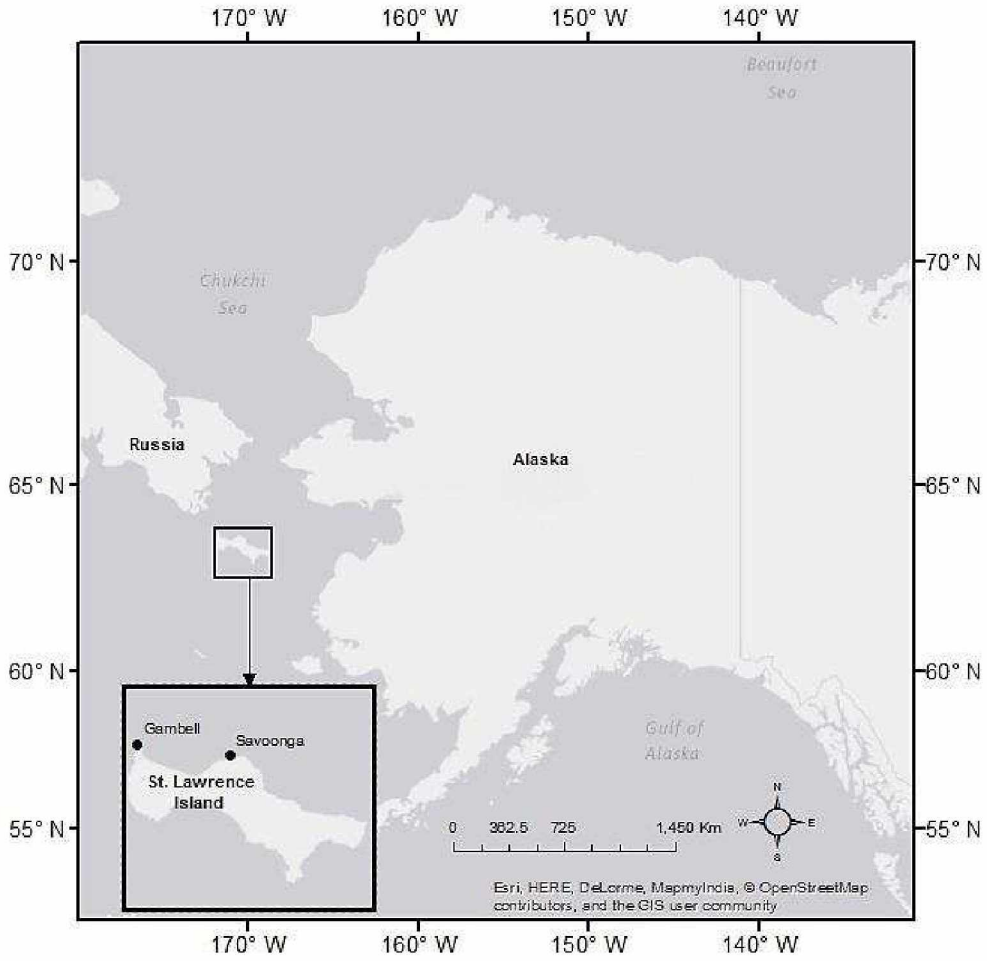


Figure 2.1: **Sampling locations on St. Lawrence Island, Alaska (AK).** Bone, blubber, and serum were collected in 2014 and 2015 from Native subsistence harvests on St. Lawrence Island, AK (inset) in the communities of Gambell and Savoonga.

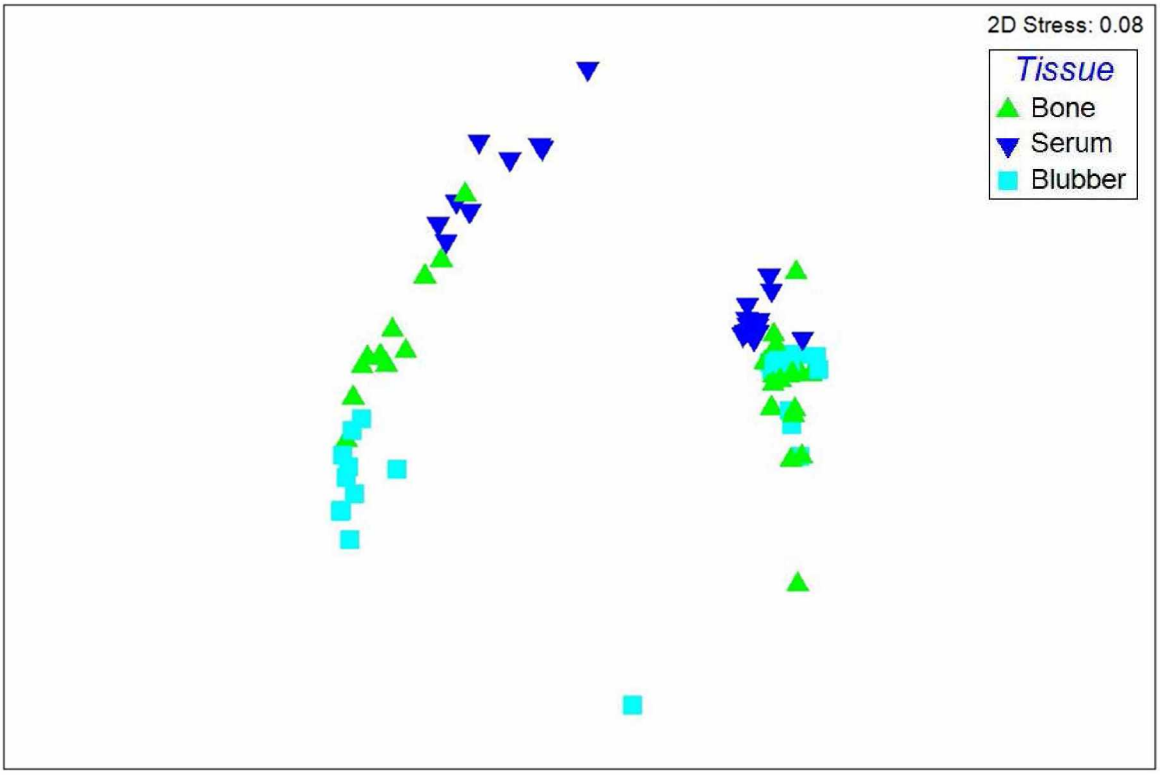


Figure 2.2: **Nonmetric dimensional scaling (nMDS) plot of walrus samples plotted by tissue.** Plot is based on similarities of cortisol, estradiol, progesterone, and testosterone concentrations plotted by tissue.

Comparison	Average Dissimilarity
2014 and 2015	85
Percent Contribution to Dissimilarity	
Estradiol	74*
Progesterone	12
Cortisol	9
Testosterone	5

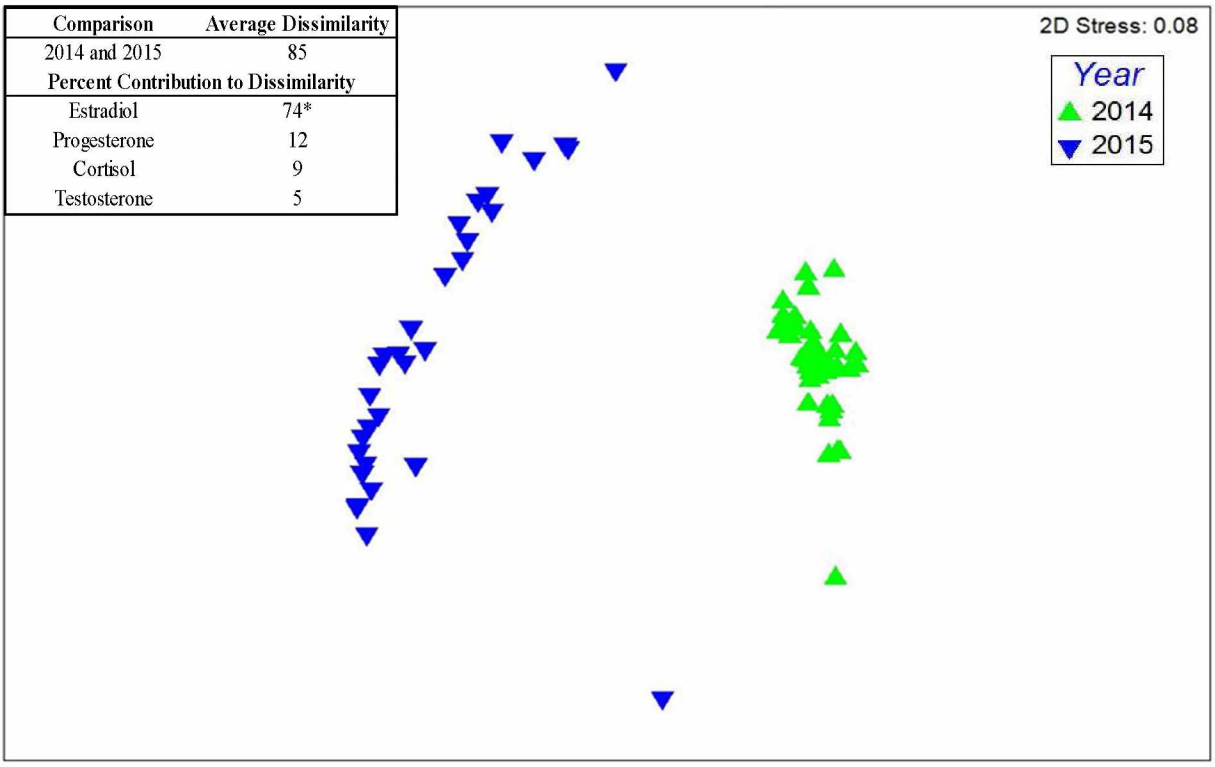


Figure 2.3: **Nonmetric dimensional scaling plot and SIMPER results of all walrus tissues plotted by year.** Plot is based on similarities among all hormone concentrations (*i.e.*, cortisol, estradiol, progesterone, and testosterone). Inset shows results from a One-Way SIMPER analysis determining which differences among hormone concentration contributed to the average dissimilarity between years. "*" notes estradiol concentration differences among samples contributed the majority to the variation between years.

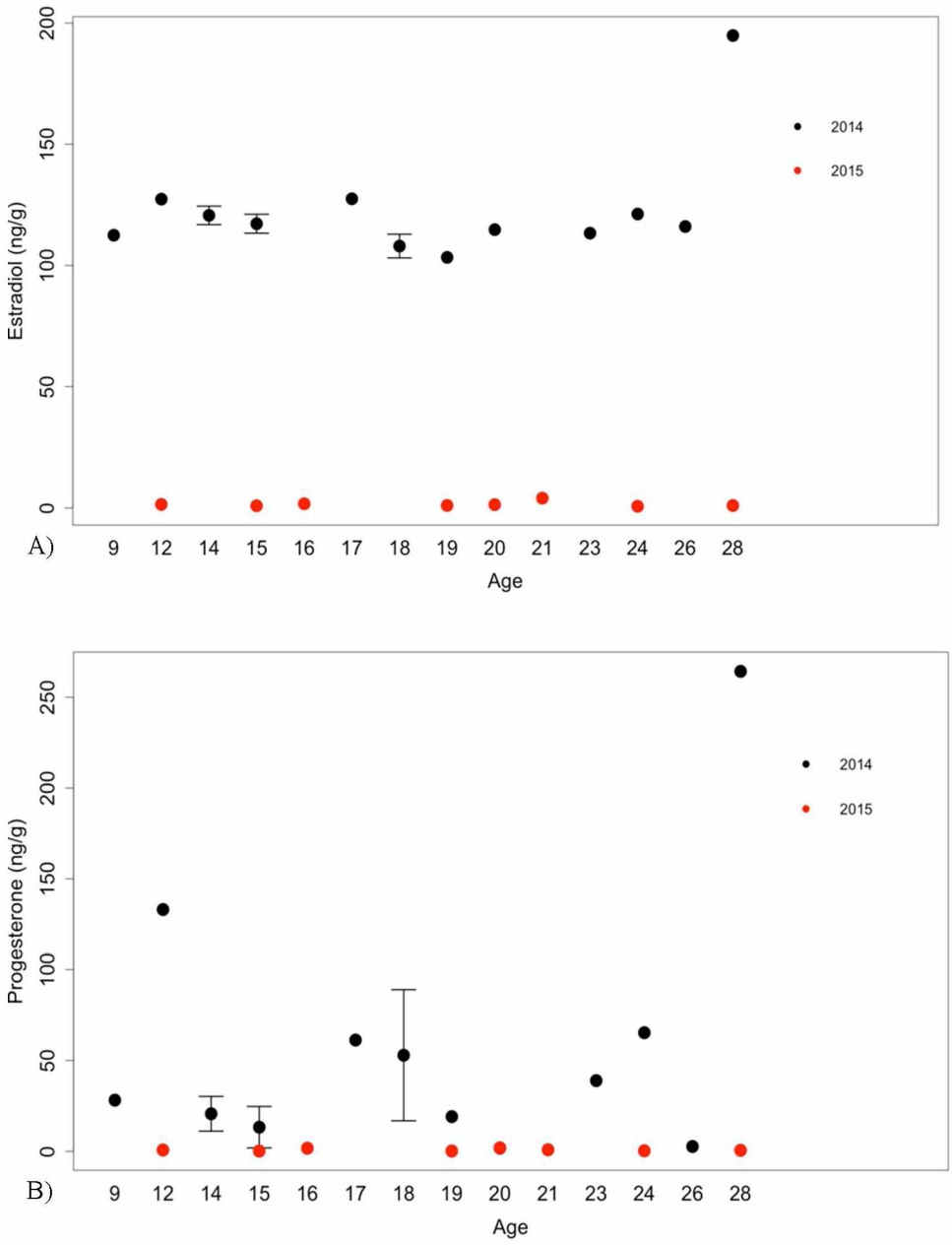


Figure 2.4 A-B: **Bone estradiol (A) and progesterone (B) concentrations of male walruses plotted by age and year collected.** Points with no error bars represent one male of that specific age from the specific year sampled. Points with error bars are the mean concentrations ± 1 standard error (SE) of males with the same age and collection year. This illustrates the high variability of estradiol measured in bone between years that is not exhibited by other hormones measured in this study (*e.g.*, progesterone). Note: only bone concentrations (ng/g, $n = 24$) are plotted and are not on a log transformed scale.

2.9 Tables

Table 2.1: **Mean, median (lipid corrected), and ranges of hormone concentrations for walrus tissues and results of ANOVAs among hormone concentrations.** In addition, the means ± 1 SD, median bone concentrations in ng/g lipid (reference purposes only), concentration ranges (non-lipid corrected), and sample sizes (*n*). Significant (bolded) *P* values are from the ANOVAs testing differences among mean log transformed steroid hormone concentrations (cortisol, progesterone, and testosterone) using two main factors (*i.e.*, sex and tissue) and an interaction term (*i.e.*, sex*tissue). If the tissue factor or the interaction term was significant, relevant *P* values (bolded if significant) from the Tukey *post hoc* tests are reported. Only female blubber progesterone concentrations were significantly different compared with male walrus tissues relating to the interaction term (*i.e.*, sex*tissue), thus only those significant *P* values are reported. Second set of *P* values for progesterone are results of ANOVA without the 2014 pregnant female walrus potentially skewing initial ANOVA progesterone results.

Sex	Tissue [Units]	Sample Size (*n*)	Hormone	Mean ± 1 SD Median [ng/g lipid]	Range (Min – Max)	*P* value (Sex)	*P* value (Tissue)	*P* value (Tissue, Tukey Post Hoc)	*P* value (Sex*Tissue)	*P* value (Sex*Tissue, Tukey Post Hoc)
Males	Bone [ng/g]	28	Cortisol	9.78 ± 22.51 67.15	0.22 - 118.84	0.06	**<0.001**	(Blubber, 0.96) (**Serum, <0.001**)	0.14	-
			Progesterone	31.67 ± 57.35 201.48	0.17 - 264.20	**(<0.001, 0.002)**	(0.27, 0.28)	-	**(0.009, 0.04)**	(**Male:Bone*Female:Blubber, 0.007, 0.04**)
			Testosterone	13.25 ± 11.53 255.20	2.18 - 64.48	1.0	**0.005**	(**Blubber, 0.003**) (Serum, 0.26)		-
	Blubber [ng/g]	27	Cortisol	4.35 ± 3.31	0.66 - 13.17	0.06	**<0.001**	(Bone, 0.96) (**Serum, <0.001**)	0.14	-
			Progesterone	4.47 ± 3.58	0.89 - 15.71	**(<0.001, 0.002)**	(0.27, 0.28)	-	**(0.009, 0.04)**	(**Male:Blubber*Female:Blubber, <0.001, 0.003**)
			Testosterone	8.17 ± 7.01	0.54 - 24.62	1.0	**0.005**	(**Bone, 0.003**) (Serum, 0.33)		-
	Serum [ng/mL]	16	Cortisol	20.80 ± 7.07	10.68 - 33.39	0.06	**<0.001**	(**Bone, <0.001**) (**Blubber, <0.001**)	0.14	-
			Progesterone	5.45 ± 5.40	0.92 - 20.46	**(<0.001, 0.002)**	0.27	-	**(0.009, 0.04)**	(**Male:Serum*Female:Blubber, 0.0011, 0.009**)
			Testosterone	8.50 ± 3.05	4.96 - 14.79	1.0	**0.005**	(Bone, 0.26) (Blubber, 0.33)	0.75	-
Females	Bone [ng/g]	6	Cortisol	4.29 ± 6.25 46.35	0.73 - 16.90	0.06	**<0.001**	(Blubber, 0.96) (**Serum, <0.001**)	0.14	-
			Progesterone	12.67 ± 5.80 246.58	6.18 - 21.31	**(<0.001, 0.002)**	(0.27, 0.28)	-	**(0.009, 0.04)**	**Reported Above**
			Testosterone	16.72 ± 19.62 188.72	5.25 - 56.21	1.0	**0.005**	(**Blubber, 0.003**) (Serum, 0.26)	0.75	-
	Blubber [ng/g]	5	Cortisol	6.15 ± 2.65	2.42 - 8.55	0.06	**<0.001**	(Bone, 0.96) (**Serum, <0.001**)	0.14	-
			Progesterone	85.50 ± 45.44	21.23 - 141.98	**(<0.001, 0.002)**	(0.27, 0.28)	-	**(0.009, 0.04)**	**Reported Above**
			Testosterone	8.26 ± 5.74	1.02 - 16.79	1.0	**0.005**	(**Bone, 0.003**) (Serum, 0.33)	0.75	-
	Serum [ng/mL]	6	Cortisol	49.41 ± 24.89	23.35 - 80.37	0.06	**<0.001**	(**Bone, <0.001**) (**Blubber, <0.001**)	0.14	-
			Progesterone	7.22 ± 5.54	3.24 - 18.04	**(<0.001, 0.002)**	(0.27, 0.28)	-	**(0.009, 0.04)**	**Reported Above**
			Testosterone	6.88 ± 2.95	2.87 - 10.61	1.0	**0.005**	(Bone, 0.26) (Blubber, 0.33)	0.75	-

Table 2.2: **Mean, median (lipid corrected), and ranges of estradiol concentrations for male and female walrus tissues and results of ANOVAs.** Estradiol concentrations of all tissues (*i.e.*, bone, blubber, and serum) ± 1 SD, median bone concentrations in ng/g lipid (for reference purposes only), concentration ranges (not lipid corrected for bone), and sample sizes (*n*) harvested in 2014 and 2015. Significant (bolded) *P* values are from ANOVAs testing differences among mean log transformed estradiol concentrations using two main factors (*i.e.*, sex and tissue) and an interaction term (*i.e.*, sex*tissue) for separate sampling years. If the tissue factor or the interaction term was significant, relevant *P* values (bolded if significant) from the Tukey *post hoc* tests are reported.

Year	Sex	Hormone	Tissue [Units]	Sample Size (*n*)	Mean ± 1 SD Median [ng/g lipid]	Range (Min - Max)	**P value (Year)	P value (Sex)	P value (Tissue)	P value (Tissue, Tukey *Post Hoc*)	P value (Sex*Tissue)
2014	Males	Estradiol	Bone [ng/g]	18	118.85 ± 20.69 100.87	100.31 - 194.85	**<0.001**	0.06	**<0.001**	(Blubber, 0.51) (**Serum, <0.001**) (Bone, 0.51) (**Serum, <0.001**) (**Bone, <0.001**) (**Blubber, <0.001**)	0.96
			Blubber [ng/g]	17	124.73 ± 16.74	106.93 - 171.88					
			Serum [ng/mL]	8	98.83 ± 25.34	83.57 - 160.44					
	Females	Estradiol	Bone [ng/g]	5	114.78 ± 8.82 2383.13	100.57 - 124.14	**<0.001**	0.06	**<0.001**	(Blubber, 0.51) (**Serum, <0.001**) (Bone, 0.51) (**Serum, <0.001**) (**Bone, <0.001**) (**Blubber, <0.001**)	0.96
			Blubber [ng/g]	4	118.29 ± 6.71	112.96 - 127.60					
			Serum [ng/mL]	5	91.89 ± 6.39	82.91 - 99.75					
2015	Males	Estradiol	Bone [ng/g]	10	1.43 ± 0.96 23.96	0.65 - 4.01	**<0.001**	-	0.38	-	-
			Blubber [ng/g]	10	1.21 ± 0.51	0.89 - 2.35				-	-
			Serum [ng/mL]	8	1.83 ± 1.28	0.77 - 4.60				-	-
	Females	Estradiol	Bone [ng/g]	1	1.44 *29.79	-	**<0.001**	-	0.38	-	-
			Blubber [ng/g]	1	0.97	-				-	-
			Serum [ng/mL]	1	1.34	-				-	-

*Due to only one female, value represents her estradiol concentrations [ng/g bone powder] in [ng/g lipid]

**Since estradiol concentrations were significantly different between years, ensuing ANOVAs were performed seperately for each year

Table 2.3: **Estimated walrus cortical bone turnover rate based on available walrus and human skeletal information.** "Calculation (Item Letters)" column shows mathematical calculations used. Dash "-"indicates no calculations were used and information for male and female walruses came from the literature "Source". Dash, "-", in the "Source" column indicates that no source was used and a mathematical calculation was done for the specific biological information.

Item	Walrus Biological Information	Calculation (Item Letters)	Male	Female	Source
A	Total Weight [kg]	-	1391.00	774.50	Fay 1982*
B	Skeleton [% of body weight]	-	4.30	3.60	Fay 1982**
C	Skeleton Weight [kg]	A*B	59.81	27.88	-
D	Cortical Bone in Walrus [kg]	0.75*C	44.86	20.91	Clarke 2008 ***
E	Cortical Bone Turnover Rate [%/year]	-	3.00	3.00	Clarke 2008****
F	Cortical Bone Turnover Rate in Walrus [kg/year]	D*0.03 (E)	1.35	0.63	-
G	**Time for Complete Cortical Bone Turnover in Walrus (years)**	D/F	**33.33**	**33.33**	-

*Median weight of adults
**One skeleton weighed for each sex - skeleton is skull with tusks and post cranial skeleton that has been thoroughly cleaned and dried
***Based on human adult skeleton - cortical bone comprises ~75% of the skeleton
****Based on human adult

Table 2.4: **Female walruses sampled with their respective provenience data, reproductive information, and steroid hormone concentrations from all tissues sampled.** Provenience data includes catalog number, sex, year collected, location, and age class. Reproductive information includes if a female has a calf and/or yearling present, is lactating and/or pregnant when sampled, with yes (Y) or no (N) indicated. Hormone abbreviations are as follows: cortisol (C), estradiol (E), progesterone (P), and testosterone (T) measured in bone, blubber, and serum. Note: Provenience data for the same (n = 6) females are on top and bottom of table with cortisol (C) and estradiol (E) concentrations from all tissues sampled located on top portion of table, while the bottom portion contains the progesterone (P) and testosterone (T) concentrations from all tissues sampled. "-" indicates that specific tissue was not collected from that individual and thus no data are available.

Catalog ID	Sex	Calf and/or Yearling Present	Lactating	Pregnant	Year Collected	Location	Age Class	Bone C [ng/g]	Blubber C [ng/g]	Serum C [ng/mL]	Bone E [ng/g]	Blubber E [ng/g]	Serum E [ng/mL]
G15-005	Female	Yearling	Y	N	2015	Gambell	Adult	2.85	2.42	67.72	1.44	0.97	1.34
G14-0046	Female	Calf	Y	N	2014	Gambell	Adult	0.73	4.35	80.37	115.22	113.84	91.49
G14-0011	Female	None	N	Y	2014	Gambell	Adult	1.63	7.48	33.08	124.14	127.60	82.91
S14-0011	Female	None	N	N	2014	Savoonga	Unknown	2.85	7.97	23.35	100.57	112.96	89.53
S14-0045	Female	Calf and Yearling	Y	N	2014	Savoonga	Adult	0.76	8.55	66.39	114.61	118.75	95.75
G14-0002	Female	Calf	Y	N	2014	Gambell	Adult	16.91	-	25.53	119.38	-	99.75

Catalog ID	Sex	Calf and/or Yearling Present	Lactating	Pregnant	Year Collected	Location	Age Class	Bone P [ng/g]	Blubber P [ng/g]	Serum P [ng/mL]	Bone T [ng/g]	Blubber T [ng/g]	Serum T [ng/mL]
G15-005	Female	Yearling	Y	N	2015	Gambell	Adult	6.18	110.87	18.04	5.25	1.02	2.87
G14-0046	Female	Calf	Y	N	2014	Gambell	Adult	10.13	85.07	4.40	6.72	7.57	7.68
G14-0011	Female	None	N	Y	2014	Gambell	Adult	7.69	141.98	4.40	13.85	16.79	4.64
S14-0011	Female	None	N	N	2014	Savoonga	Unknown	21.31	21.23	5.24	56.21	9.68	10.61
S14-0045	Female	Calf and Yearling	Y	N	2014	Savoonga	Adult	17.02	68.35	3.24	6.89	6.24	5.96
G14-0002	Female	Calf	Y	N	2014	Gambell	Adult	13.72	-	8.00	11.36	-	9.52

Table 2.5: **Summary of linear regression analysis determining correlations among walrus hormone concentrations measured in different tissues.** Bolded "*P* values" indicate a significant correlation among the same steroid hormone concentrations (*i.e.*, cortisol, estradiol, progesterone, and testosterone) measured in different tissues (*i.e.*, bone, blubber, and serum). If the regression analysis was significant (*i.e.*, $P < 0.05$), the respective "Best Fit Regression" and "R^2" values were bolded. Female (F) and male (M) samples were pooled for cortisol, estradiol, and testosterone analyses, but not for progesterone due to a significant interaction term between sex and tissue.

*Linear Model Tested	Tissues Compared	Sample Size (*n*)	Sex	Hormone Tested (Year)	*P* value	*Best Fit Regression	R^2
$Log_{10}BC \sim Log_{10}BLC$	Bone x Blubber	32	M and F		0.99	$Log_{10}BC = 0.50 + (-0.0042 \times Log_{10}BLC)$	<0.001
$Log_{10}BC \sim Log_{10}SC$	Bone x Serum	22	M and F	Cortisol	0.05	$Log_{10}BC = 1.8 + (-0.95 \times Log_{10}SC)$	0.17
$Log_{10}BLC \sim Log_{10}SC$	Blubber x Serum	20	M and F		0.46	$Log_{10}BLC = 0.057 + (0.28 \times Log_{10}SC)$	0.03
$Log_{10}BE \sim Log_{10}BLE$	Bone x Blubber	21	M and F		0.82	$Log_{10}BE = 2.2 + (-0.060 \times Log_{10}BLE)$	0.06
$Log_{10}BE \sim Log_{10}SE$	Bone x Serum	13	M and F	Estradiol (2014)	0.31	$Log_{10}BE = 1.8 + (0.14 \times Log_{10}SE)$	0.09
$Log_{10}BLE \sim Log_{10}SE$	Blubber x Serum	11	M and F		0.81	$Log_{10}BLE = 2.2 + (-0.043 \times Log_{10}SE)$	0.007
Log10BE~Log10BLE	Bone x Blubber	11	M and F		0.67	$Log_{10}BE = 0.09 + (0.21 \times Log_{10}BLE)$	0.02
Log10BE~Log10SE	Bone x Serum	9	M and F	Estradiol (2015)	0.86	$Log_{10}BE = 0.12 + (-0.060 \times Log_{10}BLE)$	0.005
Log10BLE~Log10SE	Blubber x Serum	9	M and F		0.76	$Log_{10}BE = 0.39 + (-0.060 \times Log_{10}BLE)$	0.02
$Log_{10}BP \sim Log_{10}BLP$	Bone x Blubber	5	F		**0.06	**$Log_{10}BP = -0.16 + (1.1 \times Log_{10}BLP)$**	**0.75**
$Log_{10}BP \sim Log_{10}SP$	Bone x Serum	6	F	Progesterone	0.26	$Log_{10}BP = 1.4 + (-0.42 \times Log_{10}SP)$	0.30
$Log_{10}BLP \sim Log_{10}SP$	Blubber x Serum	5	F		0.74	$Log_{10}BLP = 1.7 + (0.23 \times Log_{10}SP)$	0.04
$Log_{10}BP \sim Log_{10}BLP$	Bone x Blubber	27	M		**<0.001**	**$Log_{10}BP = -0.047 + (1.7 \times Log_{10}SP)$**	**0.51**
$Log_{10}BP \sim Log_{10}SP$	Bone x Serum	16	M	Progesterone	0.51	$Log_{10}BP = 0.22 + (0.40 \times Log_{10}SP)$	0.03
$Log_{10}BLP \sim Log_{10}SP$	Blubber x Serum	15	M		0.48	$Log_{10}BLP = 0.52 + (-0.21 \times Log_{10}SP)$	0.04
$Log_{10}BT \sim Log_{10}BLT$	Bone x Blubber	32	M and F		0.093	$Log_{10}BT = 0.86 + (0.23 \times Log_{10}BLT)$	0.06
$Log_{10}BT \sim Log_{10}ST$	Bone x Serum	22	M and F	Testosterone	0.35	$Log_{10}BT = 0.68 + (0.34 \times Log_{10}ST)$	0.04
$Log_{10}BLT \sim Log_{10}ST$	Blubber x Serum	20	M and F		0.07	$Log_{10}BLT = -0.30 + (1.1 \times Log_{10}ST)$	0.18

*Abbreviations for "Linear Model Tested" and "Best Fit Regression" were as follows: Bone (B), Blubber (BL), Serum (S) with steroid hormone concentrations being abbreviated as Cortisol (C), Estradiol (E), Progesterone (P), Testosterone (T)

**While not statistically significant, the high R^2 could be an artifact of sample size

Appendix 2.1: **List of all samples collected, tissues analyzed for steroid hormone analysis with relevant provience and steroid hormone data.** "-" indicates no data. Age class and sex determined from hunter observations. Estimated ages were based on counting cementum growth layers in the walrus teeth. Hormones are abbreviated as follows: cortisol (C), estradiol (E), progesterone (P), and testosterone (T). Tissues are abbreviated as follows: bone (B), blubber (Bl), and serum (S).

Catalog Name	Tissues Analyzed	Sex	Date Collected [D-M-Y]	Location	Age Class	Estimated Age [Years]	BC [ng/g]	BE[ng/g]	BP [ng/g]	BT [ng/g]
G14-0002	Bone, Serum	Female	25-May-14	Gambell	Adult	14	16.91	119.38	13.72	11.36
G14-0005	Bone, Blubber, Serum	Male	4-May-14	Gambell	Adult	-	118.84	100.84	26.01	19.16
G14-0011	Bone, Blubber, Serum	Female	17-May-14	Gambell	Adult	15	1.63	124.14	7.69	13.85
G14-0036	Bone, Blubber	Male	4-May-14	Gambell	Adult	14	0.77	124.46	11.14	4.84
G14-0046	Bone, Blubber, Serum	Female	4-May-14	Gambell	Adult	-	0.73	115.22	10.13	6.72
G15-005	Bone, Blubber, Serum	Female	7-May-15	Gambell	Adult	19 – 20	2.85	1.44	6.18	5.25
G15-015	Bone, Blubber, Serum	Male	15-May-15	Gambell	Adult	27 – 29	1.76	0.97	0.58	3.32
G15-023	Bone, Blubber, Serum	Male	15-May-15	Gambell	Adult	20	1.95	1.33	1.95	7.42
S14-0002a	Bone, Serum	Male	16-May-14	Savoonga	Adult	18	1.38	106.58	8.59	5.66
S14-0005	Bone, Blubber	Male	22-May-14	Savoonga	Subadult	24	3.34	121.22	65.31	23.14
S14-0007	Bone, Blubber, Serum	Male	2014	Savoonga	Unknown	18	3.61	117.06	25.75	17.18
S14-0011	Bone, Blubber, Serum	Female	22-May-14	Savoonga	Unknown	-	2.85	100.57	21.31	56.21
S14-0014	Bone, Blubber	Male	5-May-14	Savoonga	Unknown	-	8.20	107.96	10.89	12.37
S14-0018	Bone, Blubber	Male	22-May-14	Savoonga	Adult	9	32.37	112.48	28.20	15.24
S14-0021	Bone, Blubber	Male	22-May-14	Savoonga	Unknown	26	0.22	116.05	2.68	2.18
S14-0022	Bone, Blubber, Serum	Male	2014	Savoonga	Subadult	15	0.24	121.12	1.87	7.09
S14-0024	Bone, Blubber	Male	4-May-14	Savoonga	Subadult	28	0.36	194.85	264.20	64.48
S14-0029	Bone, Blubber, Serum	Male	24-May-14	Savoonga	Adult	19	8.00	103.32	19.12	14.61
S14-0034	Bone, Blubber, Serum	Male	22-May-14	Savoonga	Adult	15	6.54	113.30	24.72	16.19
S14-0035	Bone, Blubber	Male	11-May-14	Savoonga	Adult	14	22.14	116.85	30.26	18.25
S14-0036	Bone, Blubber, Serum	Male	22-May-14	Savoonga	Adult	12	2.91	127.36	133.10	15.90
S14-0038	Bone, Blubber	Male	4-May-14	Savoonga	Adult	18	1.88	100.31	124.37	13.52
S14-0039	Bone, Blubber	Male	4-May-14	Savoonga	Adult	17	3.15	127.49	61.25	12.31
S14-0040	Bone, Blubber, Serum	Male	4-May-14	Savoonga	Adult	20	2.61	114.77	1.77	3.99
S14-0044	Bone, Blubber	Male	4-May-14	Savoonga	Adult	23	11.12	113.32	38.93	15.05
S14-0045	Bone, Blubber, Serum	Female	4-May-14	Savoonga	Adult	16	0.76	114.61	17.02	6.89
S15-009	Bone, Blubber, Serum	Male	7-May-15	Savoonga	Adult	20 – 21	7.42	4.01	0.87	14.50
S15-013	Bone, Blubber, Serum	Male	11-May-15	Savoonga	Adult	15 – 16	6.82	1.68	1.73	12.26
S15-022	Bone, Blubber, Serum	Male	9-May-15	Savoonga	Adult	19	2.13	0.99	0.18	4.84
S15-027	Bone, Blubber	Male	2015	Savoonga	Unknown	-	13.60	1.44	1.19	19.00
S15-030	Bone, Blubber, Serum	Male	10-May-15	Savoonga	Adult	15	2.86	0.85	0.17	6.55
S15-036	Bone, Blubber, Serum	Male	11-May-15	Savoonga	Adult	23 – 24	2.38	0.65	0.31	6.39
S15-037	Bone, Blubber	Male	9-May-15	Savoonga	Adult	-	3.68	0.97	0.86	6.55
S15-039	Bone, Blubber, Serum	Male	10-May-15	Savoonga	Adult	11 – 13	3.48	1.42	0.74	9.01

Appendix 2.1 continued: Note: continuation of hormone concentrations analyzed in tissues only.

Catalog Name	Tissues Analyzed	Sex	Date Collected [D-M-Y]	Location	Age Class	Estimated Age [Years]	BlP [ng/g]	BlT [ng/g]	BlC [ng/g]	BlE [ng/g]
G14-0002	Bone, Serum	Female	25-May-14	Gambell	Adult	14	-	-	-	-
G14-0005	Bone, Blubber, Serum	Male	4-May-14	Gambell	Adult	-	8.41	5.33	3.59	116.77
G14-0011	Bone, Blubber, Serum	Female	17-May-14	Gambell	Adult	15	141.98	16.79	7.48	127.60
G14-0036	Bone, Blubber	Male	4-May-14	Gambell	Adult	14	3.01	19.84	6.35	121.96
G14-0046	Bone, Blubber, Serum	Female	4-May-14	Gambell	Adult	-	85.07	7.57	4.35	113.84
G15-005	Bone, Blubber, Serum	Female	7-May-15	Gambell	Adult	19 – 20	110.87	1.02	2.42	0.97
G15-015	Bone, Blubber, Serum	Male	15-May-15	Gambell	Adult	27 – 29	0.97	0.54	1.51	0.97
G15-023	Bone, Blubber, Serum	Male	15-May-15	Gambell	Adult	20	0.89	1.65	0.83	0.89
S14-0002a	Bone, Serum	Male	16-May-14	Savoonga	Adult	18	-	-	-	-
S14-0005	Bone, Blubber	Male	22-May-14	Savoonga	Subadult	24	6.44	3.82	10.74	137.98
S14-0007	Bone, Blubber, Serum	Male	2014	Savoonga	Unknown	18	6.87	1.81	5.09	151.69
S14-0011	Bone, Blubber, Serum	Female	22-May-14	Savoonga	Unknown	-	21.23	9.68	7.97	112.96
S14-0014	Bone, Blubber	Male	5-May-14	Savoonga	Unknown	-	9.89	20.95	8.39	171.88
S14-0018	Bone, Blubber	Male	22-May-14	Savoonga	Adult	9	4.04	12.52	13.17	126.28
S14-0021	Bone, Blubber	Male	22-May-14	Savoonga	Unknown	26	3.92	11.18	3.69	129.48
S14-0022	Bone, Blubber, Serum	Male	2014	Savoonga	Subadult	15	5.57	14.20	5.74	116.33
S14-0024	Bone, Blubber	Male	4-May-14	Savoonga	Subadult	28	3.27	24.62	4.47	109.51
S14-0029	Bone, Blubber, Serum	Male	24-May-14	Savoonga	Adult	19	5.06	21.59	5.98	106.93
S14-0034	Bone, Blubber, Serum	Male	22-May-14	Savoonga	Adult	15	2.95	12.60	2.34	111.35
S14-0035	Bone, Blubber	Male	11-May-14	Savoonga	Adult	14	5.39	12.03	4.29	110.98
S14-0036	Bone, Blubber, Serum	Male	22-May-14	Savoonga	Adult	12	5.48	8.12	5.30	125.22
S14-0038	Bone, Blubber	Male	4-May-14	Savoonga	Adult	18	3.51	5.27	4.00	112.36
S14-0039	Bone, Blubber	Male	4-May-14	Savoonga	Adult	17	15.71	7.66	5.96	132.76
S14-0040	Bone, Blubber, Serum	Male	4-May-14	Savoonga	Adult	20	7.43	8.80	5.29	118.63
S14-0044	Bone, Blubber	Male	4-May-14	Savoonga	Adult	23	9.14	6.50	10.12	120.34
S14-0045	Bone, Blubber, Serum	Female	4-May-14	Savoonga	Adult	16	68.35	6.24	8.55	118.75
S15-009	Bone, Blubber, Serum	Male	7-May-15	Savoonga	Adult	20 – 21	0.99	2.04	1.58	0.99
S15-013	Bone, Blubber, Serum	Male	11-May-15	Savoonga	Adult	15 – 16	5.61	2.26	2.07	1.05
S15-022	Bone, Blubber, Serum	Male	9-May-15	Savoonga	Adult	19	0.98	3.37	0.72	0.98
S15-027	Bone, Blubber	Male	2015	Savoonga	Unknown	-	1.07	1.77	1.13	1.97
S15-030	Bone, Blubber, Serum	Male	10-May-15	Savoonga	Adult	15	0.90	1.68	0.82	0.90
S15-036	Bone, Blubber, Serum	Male	11-May-15	Savoonga	Adult	23 – 24	0.99	1.73	2.55	0.99
S15-037	Bone, Blubber	Male	9-May-15	Savoonga	Adult	-	1.04	4.20	1.03	1.04
S15-039	Bone, Blubber, Serum	Male	10-May-15	Savoonga	Adult	11 – 13	1.06	4.44	0.66	2.35

Appendix 2.1: continued. Note: continuation of hormone concentrations analyzed in tissues only.

Catalog Name	Tissues Analyzed	Sex	Date Collected [D-M-Y]	Location	Age Class	Estimated Age [Years]	SP [ng/mL]	ST [ng/mL]	SC [ng/mL]	SE [ng/mL]
G14-0002	Bone, Serum	Female	25-May-14	Gambell	Adult	14	8.00	9.52	25.53	99.75
G14-0005	Bone, Blubber, Serum	Male	4-May-14	Gambell	Adult	-	5.00	9.09	12.75	83.57
G14-0011	Bone, Blubber, Serum	Female	17-May-14	Gambell	Adult	15	4.40	4.64	33.08	82.91
G14-0036	Bone, Blubber	Male	4-May-14	Gambell	Adult	14	-	-	-	-
G14-0046	Bone, Blubber, Serum	Female	4-May-14	Gambell	Adult	-	4.40	7.68	80.37	91.49
G15-005	Bone, Blubber, Serum	Female	7-May-15	Gambell	Adult	19 – 20	18.04	2.87	67.72	1.34
G15-015	Bone, Blubber, Serum	Male	15-May-15	Gambell	Adult	27 – 29	14.95	8.15	33.39	2.37
G15-023	Bone, Blubber, Serum	Male	15-May-15	Gambell	Adult	20	20.46	12.37	26.66	0.77
S14-0002a	Bone, Serum	Male	16-May-14	Savoonga	Adult	18	1.59	10.68	10.68	85.25
S14-0005	Bone, Blubber	Male	22-May-14	Savoonga	Subadult	24	-	-	-	-
S14-0007	Bone, Blubber, Serum	Male	2014	Savoonga	Unknown	18	9.21	5.20	23.80	94.33
S14-0011	Bone, Blubber, Serum	Female	22-May-14	Savoonga	Unknown	-	5.24	10.61	23.35	89.53
S14-0014	Bone, Blubber	Male	5-May-14	Savoonga	Unknown	-	-	-	-	-
S14-0018	Bone, Blubber	Male	22-May-14	Savoonga	Adult	9	-	-	-	-
S14-0021	Bone, Blubber	Male	22-May-14	Savoonga	Unknown	26	-	-	-	-
S14-0022	Bone, Blubber, Serum	Male	2014	Savoonga	Subadult	15	2.32	14.79	16.89	160.44
S14-0024	Bone, Blubber	Male	4-May-14	Savoonga	Subadult	28	-	-	-	-
S14-0029	Bone, Blubber, Serum	Male	24-May-14	Savoonga	Adult	19	3.80	14.03	19.69	89.64
S14-0034	Bone, Blubber, Serum	Male	22-May-14	Savoonga	Adult	15	4.80	9.15	14.84	94.05
S14-0035	Bone, Blubber	Male	11-May-14	Savoonga	Adult	14	-	-	-	-
S14-0036	Bone, Blubber, Serum	Male	22-May-14	Savoonga	Adult	12	3.43	7.01	23.91	86.53
S14-0038	Bone, Blubber	Male	4-May-14	Savoonga	Adult	18	-	-	-	-
S14-0039	Bone, Blubber	Male	4-May-14	Savoonga	Adult	17	-	-	-	-
S14-0040	Bone, Blubber, Serum	Male	4-May-14	Savoonga	Adult	20	0.93	7.09	27.80	96.81
S14-0044	Bone, Blubber	Male	4-May-14	Savoonga	Adult	23	-	-	-	-
S14-0045	Bone, Blubber, Serum	Female	4-May-14	Savoonga	Adult	16	3.24	5.96	66.39	95.75
S15-009	Bone, Blubber, Serum	Male	7-May-15	Savoonga	Adult	20 – 21	3.23	7.64	12.58	2.28
S15-013	Bone, Blubber, Serum	Male	11-May-15	Savoonga	Adult	15 – 16	3.00	5.38	15.01	0.93
S15-022	Bone, Blubber, Serum	Male	9-May-15	Savoonga	Adult	19	0.92	5.64	29.92	0.84
S15-027	Bone, Blubber	Male	2015	Savoonga	Unknown	-	-	-	-	-
S15-030	Bone, Blubber, Serum	Male	10-May-15	Savoonga	Adult	15	3.95	4.96	16.33	4.60
S15-036	Bone, Blubber, Serum	Male	11-May-15	Savoonga	Adult	23 – 24	1.55	7.59	19.70	1.55
S15-037	Bone, Blubber	Male	9-May-15	Savoonga	Adult	-	-	-	-	-
S15-039	Bone, Blubber, Serum	Male	10-May-15	Savoonga	Adult	11 – 13	8.00	7.18	28.85	1.33

Chapter 3: Steroid Hormone Concentrations in Pacific Walrus Bone Reveal Long-term Changes in Reproductive Status and Stress Response over the Last 3 Millennia[1]

3.1 Abstract

The Pacific walrus (*Odobenus rosmarus divergens*) is an iconic Arctic marine mammal that Alaskan Natives rely on as a food, economic, and cultural resource. A decrease in critical sea ice habitat and poorly understood population numbers have led to walruses being listed as a candidate for the Endangered Species Act. Yet, there is no clear understanding of how walruses might be affected by changing climate. In this study, steroid hormone concentrations (i.e., cortisol, estradiol, progesterone, and testosterone) were analyzed from walrus bones, spanning ~ 3450 years, to track changes in reproductive status and stress response over time. Our results show modern walrus samples from 2014 and 2015 have similar cortisol concentrations (median = 59.16 ± (SD) 1118.54 ng/g lipid) to archaeological walruses (aged 3450 years before present (BP) – 200 BP, 43.26 ± 345.32 ng/g lipid, $P = 0.38$) and historical walruses (aged 200 BP – 20 BP, 81.33 ± 1591.60 ng/g lipid, $P = 0.07$) indicating a possible physiological resiliency to the current receding sea ice in the Arctic. Progesterone and testosterone concentrations are significantly correlated with walrus population size ($r = -0.21$, $P = 0.003$ and $r = -0.29$, $P < 0.001$, respectively), and differences in progesterone and estradiol concentrations contributed the most to the variation seen among samples grouped by decades (1830s–2010s) and sample time periods (i.e., archaeological, historical, and modern). Possible local production of estradiol in

[1] Submitted to Ecology as Charapata P, Horstmann L, Misarti N "Steroid hormone concentrations in Pacific walrus bone reveal long-term changes in reproductive status and stress response over the last 3 millennia"

walrus bone indicates a relatively shorter reservoir time in cortical bone compared with other hormones, contributing to variation seen among samples from different time periods. Data from 2014 – 2015 indicate that the current walrus population has significantly lower ($P = 0.04$) reproductive hormone concentrations compared with walruses during times of rapid population increase. This may be due to low calf production and/or be indicative of a population at carrying capacity. These data provide marine mammal management with a new tool to monitor long-term changes in stress response and reproductive status that may relate to population size.

3.2 Introduction

The Pacific walrus (*Odobenus rosmarus divergens*) (hereafter referred to as walrus) is an important Arctic marine mammal that Russian and Alaskan Natives rely on for cultural, economic, and subsistence purposes (Metcalf and Robards 2008). Walruses are benthic predators foraging on bivalves and amphipods as their preferred prey (Fay 1982, Sheffield and Grebmeier 2009), although feeding on higher trophic level prey such as seals and sea birds has been documented (Lowry and Fay 1984, Seymour et al. 2014). Females, calves, and some male walruses use summer ice in the Chukchi Sea as a feeding, resting, and molting platform (Fay 1982). Walruses passively float on sea ice to different benthic feeding locations in the Chukchi Sea conserving energy that is used for other purposes (e.g., energy stores and/or reproduction, Fay 1982, Jay et al. 2012). Recently, Arctic warming has caused sea ice to recede into deep Arctic basin waters, limiting access to benthic prey due to walruses' relatively shallow dive limit of about 250m (Fay and Burns 1988, Jay and Fischbach 2008, Jay et al. 2012). Walruses have responded to this altered summer habitat by resorting to coastal land haulouts, where they may feed in less productive areas or go on energetically costly foraging trips (up to 200km one way) to Hanna Shoal (Jay et al. 2012). The reduction of critical sea ice habitat has caused an increase in trampling deaths of calves on terrestrial haulouts (Udevitz et al. 2013), calves becoming separated from their mothers (Cooper et al. 2006), and increased energy expenditure in foraging (Jay et al. 2012). Females are most affected due to the higher energy requirements needed for reproduction (Noren et al. 2014), which could explain why the population has recently started to show decreases in fecundity (Garlich-Miller et al. 2006). The Chukchi Sea remains a rich benthic foraging ground, but northerly shifts in walruses and their prey are expected due to a potential

change to a more pelagic food web (Grebmeier et al. 2006, Grebmeier 2012, Schonberg et al. 2014, Jay et al. 2014).

A change in the movement patterns of walruses in response to receding sea ice has already caused changes to Native Alaskan hunting patterns (Fidel et al. 2014). For example, hunters have to undergo longer and more dangerous hunting excursions to pursue walruses (Fidel et al. 2014). In addition, hunters from Wrangell Island are reporting more malnourished females compared to years with adequate sea ice (Metcalf and Robards 2008). Potential shortages in numbers and poor body condition of walruses harvested could cause hardships among local subsistence users. For example, in 2013, a 'state of emergency' was issued on St. Lawrence Island due to substantially reduced harvest numbers, and federal aid was provided to the communities to survive the year (Caldwell 2013). Human dependency on walruses reflects the importance of understanding the long-term health of the walrus population in response to loss of sea ice habitat. Numerous behavioral and observational studies in response to receding sea ice have been conducted and provided possible outcomes to the long-term health of the population (e.g., Cooper et al. 2006, Laidre et al. 2008, Jay et al. 2012, 2014, MacCracken 2012, Schonberg et al. 2014). However, there are limited studies on the physiological effects these changes have on the walrus population. Long-term studies such as this can determine an accurate physiological baseline of the walrus population before the reduction of sea ice habitat in the Arctic by analyzing walrus bone steroid hormone concentrations from archaeological time periods. A comparison of the baseline walrus physiological state to that of the present-day walrus population allows researchers to detect any significant physiological changes due to climate change in the Arctic.

Steroid hormones, including cortisol, estradiol, progesterone, and testosterone, can give important physiological information on marine mammals. Estradiol, progesterone, and testosterone are reproductive hormones and have been used to determine marine mammal reproductive status, including pregnancy in cetaceans (Rolland et al. 2005, Kellar et al. 2006, Trego et al. 2013, Hunt et al. 2014), pinnipeds (Pomeroy 2011), and Pacific walruses (Triggs 2013). Cortisol is an indicator of stress response, and pinnipeds with high cortisol concentrations are more susceptible to disease and may have poorer body condition (Gulland et al. 1999, Burek et al. 2008). Steroid hormone studies of marine mammals are imperative for the assessment of marine mammal population health status and their monitoring thereafter (Fair and Becker 2000).

Steroid hormones have been extracted from various matrices including feces (Rolland et al. 2005), blubber, serum, urine (Kellar et al. 2013), saliva (Atkinson et al. 1999, Triggs 2013), and whale blow (Hogg et al. 2009). These tissues give information on the acute stress response and a snapshot of reproductive status of an individual and require repeat sampling to create a long-term hormone profile. Assessing acute stress response via serum can also be distorted by chase and capture sampling techniques, thus skewing a "baseline" stress response by artificially inducing high cortisol concentrations (Fair and Becker 2000). This type of capture stress response has been documented in serum of Weddell seals (*Letonychotes weddellii*, Harcourt et al. 2010) and in some cetaceans (Thomson and Geraci 1986, Fair et al. 2014, but see Kjeld (2001) for fin whale (*Balaenoptera physalus*)). Additionally, it is difficult to sample steroid hormones from free-ranging marine mammal populations that seasonally migrate thousands of miles or have remote distributions.

Advances have been made in assessing long-term stress response and reproductive profiles of marine mammals, including cortisol and progesterone from baleen of bowhead whales (*Balaena mysticetus*, Hunt et al. 2014) and utilizing earplugs from blue whales (*Balaenoptera musculus*) to create lifetime testosterone and cortisol hormone profiles (Trumble et al. 2013). Asymmetry of walrus tusks has also been analyzed as a proxy for stress (MacCracken and Benter 2016). However, there are limitations to these tissues, as tusks, baleen, and earplugs are only found in some marine mammals, limiting these analyses to those species. In addition, baleen only provides hormone data over a fraction of the lifespan (~10 years) of a baleen whale (George and Bockstoce 2008).

Bone tissue contains lipids, which are sequestered over the lifespan of an animal, do not significantly degrade after death, and are detectable in the bone after being buried for thousands of years (Evershed et al. 1995, During et al. 2015). Cortisol, estradiol, progesterone, and testosterone are lipophilic steroid hormones (Norris 1997) making extraction of both reproductive and stress hormones from bone possible. Testosterone and estrogens, including estradiol, have been extracted and used to sex human bones as old as 6961 calendar years before present (BP) (Mark et al. 2011), and testosterone and estradiol have been extracted and analyzed from rat bone (Yarrow et al. 2010). Bone has a slow turnover rate (3% cortical bone/year, Clarke 2008), so, hormone concentrations from bone could represent a lifelong accumulated average of an individual. This is beneficial when monitoring long-term physiological changes in a population, because bone hormone concentrations are not likely skewed by acute stress or reproductive events. While lipids have been obtained from ancient whale bone (75000 BP, Evershed et al. 1995), steroid hormones have not yet been extracted from marine mammal bones.

The modern walrus population is not an ideal baseline for studying the physiological resilience (i.e., reproductive status and stress response) to receding sea ice, because significant reduction of sea ice over the Chukchi Sea's shelf has occurred over at least the past nine years (Jay et al. 2012). Thus, monitoring acute stress and reproductive response with serum and blubber hormone studies of current and future walrus populations would be an inaccurate portrayal of the physiological resilience to climate change in the Arctic. The physiological status of the archaeological walrus population would provide a more precise baseline. During archaeological times related to this study (3450 – 200 BP), the Arctic climate was significantly different from the industrial modern Arctic climate (275 BP – present, IPCC 2013). After the early Holocene Thermal Maximum (11000 – 7000 BP), which included Arctic seasonal sea ice free periods, a cooling period began known as Neoglacial cooling (Cronin and Cronin 2015). This Neoglacial cooling period was interrupted with natural warming anomalies, including the Medieval Climate Anomaly (1215 BP – 700 BP), with the minimum preindustrial Arctic sea ice extent occurring before this warming period around 1375 BP (Kinnard et al. 2011). Recently (1950 AD – present), there has been a reversal in the Arctic cooling trend attributed to in part anthropogenic greenhouse emissions (Kaufman et al. 2009). Possible advection of unprecedented warm Atlantic water into the Arctic is a factor in the rapid sea ice decreases observed today in the Arctic (Spielhagen et al. 2011). Thus, today's walrus population potentially has to adapt at a faster rate than in the past, making walruses from archaeological time periods an accurate physiological baseline to compare with the physiology of present-day walruses. This comparison can help determine how physiologically resilient walruses are to the current rapid climate change occurring in the Arctic today.

In this study, steroid hormone concentrations from archaeological, historical (200 – 20 BP), and modern (2014 – 2015) walrus bone are analyzed to investigate the reproductive status (i.e., changes in estradiol, progesterone, and testosterone concentrations) and stress response (i.e., changes in cortisol concentrations) of walruses. The objectives of this study are to: 1) validate a method of lipid extraction and liquid chromatography tandem mass spectrometry (LC/MS/MS) for analyzing steroid hormone concentrations from archaeological, historical, and modern walrus bone; 2) compare "true" baseline stress response of archaeological bone cortisol concentrations with modern bone cortisol concentrations; 3) determine if reproductive hormones are correlated with walrus population size through time; and 4) establish the reproductive status of the modern walrus population. Secondary objectives of this study are to: 1) determine if sex of unknown individuals in this study can be determined via reproductive steroid hormone concentrations; and 2) investigate general trends in walrus bone steroid hormones over time. This study provides the first results of steroid hormone concentrations extracted from marine mammal bone including archaeological walrus bone as old as 3450 BP. The bone steroid hormone method and results of this study provide a new long-term tool for monitoring stress response and reproductive status of walruses and potentially other marine mammals.

3.3 Methods

3.3.1 Sample Collection

Walrus bone samples (n = 220) were collected and categorized into time periods, including archaeological (n = 38), historical (n = 135), and modern (n = 47). Bones from historical and modern time periods were further categorized into finer time periods (i.e., decades) for analysis of steroid hormone concentrations over shorter time scales. This includes the 1830s

(n = 4), 1840s (n = 8), 1870s (n = 1), 1880s (n = 5), 1890s (n = 3), 1900s (n = 7), 1910s (n = 1), 1920s (n = 2), 1930s (n = 13), 1950s (n = 28), 1960s (n = 34), 1970s (n = 28), 1980s (n = 2), and 2010s (n = 47). Samples for all time periods combined included 52 females, 94 males, and 74 unknowns.

3.3.1.1 Archaeological Samples

Archaeological walrus bones were acquired from various sites throughout the Alaskan and Russian walrus habitats (Figure 3.1, Appendix 3.1). This includes archaeological sites in Barrow, Nome, Point Hope, Point Lay, Port Moller, and Sanak Island, Alaska (AK) (Figure 3.1). Walrus bones from archaeological sites were obtained through the University of Alaska Museum (UAM) Archaeological Collection, Dr. A. Jensen, at Ukpeaġvik Iñupiat Corporation (UIC) in Barrow, Alaska, and other archaeological excavations in Port Moller and Sanak Island, AK. The minimum number of individuals (MNI) was determined by selecting the largest number of the most common bone element located in each site to ensure that each bone sampled was an individual (Grayson 1978). Sites that provide additional detailed location information relating to where each bone was collected such as depth (e.g., 5 cm vs 15 cm) and/or unit (e.g., north quadrant vs. south quadrant of dig site), increase the chances that bone elements selected from different units and depths are not from the same individual (Grayson 1984), and thus, various bone elements were selected for those respective sites. Walrus bones were assigned an estimated age class (e.g., subadult and adult) based on a combination of size and degree of fusion between the respective element and its epiphyses when possible (Davis 1992). Portions of archaeological samples are fragments of walrus bone; therefore, bone fragments of archaeological samples were not assigned an age class.

3.3.1.2 Historical Samples

Historical samples were acquired from the UAM Mammal and Archaeological Collections and the Smithsonian Institution National Museum of Natural History. Only samples with a collection or radiocarbon date and collection location were used. The majority of samples included other provenience data, such as sex and age class. In addition, certain samples had body size, weight, and blubber thickness data. A list of samples with the respective provenience data is provided in Appendix 3.2.

3.3.1.3 Modern Samples

Modern samples were collected from Native subsistence harvests through an agreement with Native hunters, the Eskimo Walrus Commission, the Alaska Department of Fish and Game (ADF&G), and the U.S. Fish and Wildlife Service (USFWS) during May 2014 and 2015. Hunters recorded sex, age class, and reproductive information for harvested females (i.e., presence of a fetus, calf, yearling, and/or lactating). Bone samples were transferred to the University of Alaska Fairbanks for sample analysis under a Letter of Authorization to Dr. L. Horstmann. Additional modern samples were opportunistically collected from Barrow ($n = 3$) in partnership with the North Slope Borough Department of Wildlife Management and subsistence hunters from the Barrow area. A list of modern samples with provenience data (e.g., sex and age class) is provided in Appendix 3.3.

3.3.2 Ageing Archaeological Samples

Radiocarbon dating is based on the radioactive decay of the ^{14}C radioisotope of carbon in biological materials, including bone, and provides a tool for aging archaeological samples

(Bowman 1990, Cherkinsky 2009). However, direct radiocarbon dating of marine animal tissue consistently results in a pre-dating error, making marine animal tissues appear older than terrestrial animals of the same age due to the "marine reservoir" effect of ^{14}C carbon in marine environments (Dumond and Griffin 2002). Therefore, bone from caribou (*Rangifer tarandus*), a terrestrial grazer, was dated from the same site, unit, and depth as walrus utilized in this study. Bone collagen was extracted following Misarti et al. (2009) and submitted for AMS dating at the Center for Applied Isotope Studies at the University of Georgia (Cherkinsky 2009). The raw AMS dates were calibrated using a calibration curve from the CALIB ^{14}C Calibration Program (v7.1, Stuiver et al. 2005).

3.3.3 Steroid Hormone Extraction

All walrus bones, archaeological, historical, and modern, were extracted for steroid hormones following the same procedure outlined below. Sections of bone were polished with a Dremel® 3000 drill with a sand drum attachment to remove outside contaminants exposing clean areas of cortical bone. Approximately 1.5 g of cortical bone was removed for steroid hormone extraction using the Dremel drill with a diamond blade attachment. Pieces of bone were pulverized into powder using a Wig-L-Bug® and 0.2 - 0.3 g of powdered bone were transferred to 2.8 mL ceramic bead homogenizer cryovials. Samples were homogenized, dry, on a Disruptor Genie® (Scientific Industries) for one minute. Samples were spiked with 100 ng of isotopically labeled internal standards (Sigma Aldrich) (ISTD), d_4-cortisol, $^{13}C_3$-testosterone-2, d_9-progesterone, and d_5-estradiol, for accurate hormone detection and validation during LC/MS/MS analysis (Difrancesco et al. 2007, Hogg et al. 2009, Zhang et al. 2009, Koal et al. 2012, Murtagh et al. 2013). Lipid extraction of the powdered bone was done by adding 1.460 mL of methanol

(BDH®, Accorsi et al. 2008, Bryan et al. 2013, Hunt et al. 2014). Samples were homogenized for three minutes on a Disruptor Genie® (Scientific Industries) and set on a rocking platform (VWR®; Model 100) for 24 hours. Samples were then centrifuged (Microfuge® 18 Centrifuge, Beckman Coulter™) at 12000 RPMs for 20 minutes. Supernatant from each sample was pipetted into glass vials and remaining methanol was dried using nitrogen gas (N-EVAP™112, Organomation Associates, Inc.) leaving only lipids. Samples were then stored in a -80 °C freezer until analysis.

3.3.4 LC/MS/MS Analysis of Steroid Hormones

Prior to analysis, each sample was reconstituted in 200 µL of methanol, split into two equal aliquots and dried again using an Eppendorf-Vacufuge rotary evaporating device. The first aliquot of each extract was derivatized with dansyl chloride according to Zhang et al. (2009) just prior to LC/MS/MS analysis. To each sample, 20 µL of 10 mM Na_2CO_3 and 50 µL of freshly prepared dansyl chloride solution (3 mg/mL acetone) were added. The samples were heated at 60 °C for 10 minutes, transferred to autosampler vials, and immediately analyzed. The second aliquot of each extract was derivatized with the AB Sciex Keto derivatization kit (AB Sciex, Framingham, MA) just prior to LC/MS/MS analysis and 50 µL of reagent was added. The reaction time was 60 minutes at room temperature. The samples were transferred to autosampler vials and immediately analyzed.

An Agilent 1200 Rapid Resolution Liquid Chromatography (LC) system coupled to an Agilent 6460 series QQQ mass spectrometer (MS) was used to analyze all samples after derivatization at the Bindeley Bioscience Center at Purdue University, IN. For the dansyl chloride derivatives, the following conditions were used. A Waters Xbridge C18 2.1 mm x 100

mm, 3-µm column was used for LC separation. The buffers were (A) water + 0.1 % formic acid and (B) acetonitrile + 0.1 % formic acid. The linear LC gradient was as follows: time 0 minutes, 10 % B and 90 % A; time 5 minutes, 100 % B and 0 % A; time 15.5 minutes, 10 % B and 90 % A; time 18 minutes, 10 % B and 90 % A. The flow rates of buffers (A) and (B) were 0.3 mL/min. Multiple reaction monitoring was used for MS analysis. The data were acquired in positive electrospray ionization (ESI) mode by monitoring the following transitions: estradiol (dansyl Cl), *m/z* (atomic mass units) 506.1→171 (30 V), m/*z* 155.8 (40 V); d_5-estradiol (dansyl Cl), *m/z* 511.1→171 (30 V), *m/z* 155.8 (40 V); estriol (dansyl Cl), *m/z* 522→171 (30 V), 155.8 (40 V). This method can also be used to monitor progesterone in its unlabeled form by following the transition: *m/z* 315.2→109 (15 V), 97 (15 V); d_9-progesterone, *m/z* 324.2→113 (15 V), 100 (15 V) if necessary. ESI interface had a nitrogen gas temperature of 325 °C, nitrogen gas flow rate of 8 L/minute, nebulizer pressure of 45 psi, sheath gas temperature of 250 °C, sheath gas flow rate of 7 L/minute, capillary voltage of 3500 V, and nozzle voltage of 1500 V.

For the keto derivatives, the following conditions were used for LC/MS/MS analysis. An Agilent Zorbax 80Å Extend-C18 4.6 mm x 150 mm, 5-µm column was used with the buffers (A) water + 0.1 % formic acid and (B) acetonitrile + 0.1 % formic acid. The linear LC gradient was as follows: time 0 minutes, 10 % B and 90 % A; time 10 minutes, 100 % B and 0 % A; time 12 minutes, 10 % B and 90 % A; time 15 minutes, 10 % B and 90 % A. The flow rates of buffers (A) and (B) were 0.3 mL/min. Multiple reaction monitoring was used for MS analysis. The data were acquired in positive ESI mode by monitoring the following transitions: testosterone, *m/z* 403.1→344.1 (20 V), 164 (40 V); $^{13}C_3$-testosterone *m/z* 406.1→347.1 (20 V), 167 (40 V); cortisol *m/z* 477.1→418.3 (15 V), 388.2 (35 V); d_4-cortisol *m/z* 481.1→422.3 (15 V), 392.3 (35

V); progesterone *m/z* 429.1→370 (20 V), 126 (30 V); d_9-progesterone *m/z* 438.1→379 (20 V), 132 (30 V). The jet stream ESI interface had a nitrogen gas temperature of 325 °C, nitrogen gas flow rate of 8 L/minute, nebulizer pressure of 45 psi, sheath gas temperature of 250 °C, sheath gas flow rate of 7 L/minute, capillary voltage of 4000 V, and nozzle voltage of 1000 V.

Samples with hormone concentrations below detection limit for LC/MS/MS analysis (< 0.5 ng), were included in statistical analysis by assigning one-half the detection limit concentrations for each hormone with a non-detectable signal (Gilbert 1987, Dehn et al. 2005). Extraction efficiencies were determined by comparing known volumes of added ISTDs of each hormone that had been though the extraction process (i.e., blank samples that went through the steroid hormone extraction method with no bone sample, but added ISTDs and methanol, $n = 8$, "Blank-Extraction"), to samples with ISTDs and no extraction (i.e., added ISTD to vial and dried using nitrogen gas, $n = 5$, "Blank-Dried ISTDs"). The percent recovery of each ISTD was calculated by comparing the ratio of mean hormone concentration detected in "Blank-Extraction", divided by the mean hormone concentration in the "Blank-Dried ISTDs" samples. The mean extraction efficiencies for each hormone in walrus bone are as follows: progesterone = 51 %, testosterone = 107 %, cortisol = 72 %, and estradiol = 79 %.

3.3.5 Percent Lipid Correction Factor

Walrus bones from different archaeological, historical, and modern time periods potentially have different lipid compositions, as lipid in cortical bone is already low (Clarke 2008) and taphonomic processes could affect the lipid composition of archaeological bones buried for thousands of years (Evershed et al. 1995, Collins et al. 2002). A mean percent lipid correction factor was used to correct potential lipid composition differences among sample time

periods. Bones (n = 12, 10, 12, for archaeological, historical, and modern bone, respectively) from each sample time period (i.e., archaeological, historical, and modern) were lipid extracted using a modified (2:1 chloroform: methanol) Soxhlet procedure after Schlechtriem et al. (2003). A one-way Analysis of Variance (ANOVA) followed by Tukey pairwise comparison determined that mean percent lipid of modern bone (4.83 ± 1.78 %) was significantly higher than archaeological (2.71 ± 1.96 %) and historical (1.98 ± 1.52 %) walrus bone (P = 0.02, 0.002, respectively). Therefore, hormone concentrations (steroid hormones are lipophilic as described above) from all samples were corrected by mean percent lipid weight based on their sample time period and are reported as ng/g lipid.

3.3.6 Minimum Walrus Population Estimates

Minimum estimates were calculated for the Pacific walrus population from 1830 – 2015. A geometric growth rate model from Udevitz et al. (2013):

$$(3.1)\ GR=N_i/N_j^{1/(i-j)}$$

Where GR = estimated growth rate, N_i = population from year i, where year i > year j, N_j = population from year j, to determine estimated walrus population growth rates (Udevitz et al. 2013). The minimum estimates for the Pacific walrus population from 1830 – 2015 were determined by applying published estimates of the minimum walrus population (Table 3.1) to the following formula:

$$(3.2)\ P_i = P_j*GR$$

Where P_i = the minimum walrus population at year i, and P_j = the minimum walrus population the previous year in relation to P_i, and GR = the geometric growth rate of the walrus population (Udevitz et al. 2013, Table 3.2). These minimum population estimates were used to determine

any potential correlations between walrus population change and steroid hormone concentrations.

3.3.7 Estimated Bone Hormone Turnover Rate

Bone steroid hormones have not been studied in marine mammals (and only in a very limited number of studies conducted on terrestrial mammals), and therefore, the turnover of bone hormone concentrations is unknown (Yarrow et al. 2010). Lipids and the steroid hormones that are associated with them, are present in both cortical and marrow bone tissue (During et al. 2015). Lipids in cortical bone either reside in osteocytes, which are embedded in the cortical bone or are directly associated with cortical bone matrix compounds (During et al. 2015). Only cortical bone was sampled in this study. Therefore, we calculated an estimate of walrus cortical bone turnover rate in this study and applied it to steroid hormone turnover in cortical bone. The walrus cortical bone turnover rate was based on the human cortical bone turnover rate (Clarke 2008), the human cortical bone skeletal makeup (Clarke 2008), and Pacific walrus skeletal and biological information (Fay 1982, summarized in Table 3.3):

$$(3.3)\ Cw_y = ((P_s * M_w) * P_c) * (Ch_y)$$

Where Cw_y is the walrus cortical bone turnover rate (kg/year), P_s is the percent skeletal mass of total walrus mass (%), M_w is the mass of a walrus (kg), P_c is the percent cortical bone in a human skeleton (%), and Ch_y is the cortical bone turnover rate in humans (% / year). There is variation in the adult human cortical bone turnover rate of 2 - 3 % / year (Clarke 2008). However, Clarke (2008) does not give information about sex specific cortical bone turnover rates. Therefore, we chose the highest rate of 3 % / year, because this would give us a minimum reservoir time of

steroid hormones in walrus cortical bone. This turnover rate is an estimate, and it is used to argue that bone hormones are mean concentrations representing an individual's lifetime (Table 3.3). Further study is needed to confirm this bone time signature for steroid hormone concentrations.

3.3.8 Statistical Analysis

Until this study, steroid hormone concentrations had not been measured and compared in different walrus skeletal elements. Therefore, a small pilot study was performed using paired two-tailed t-tests on skull and mandible bone sampled from the same individual walrus to determine if hormone concentrations were significantly different between skeletal elements (n = 7). If steroid hormones measured in skulls and mandibles were similar, comparisons of steroid hormone concentrations in different walrus skeletal elements was accepted. All steroid hormone concentrations were similar between skulls and mandibles from the same individual (cortisol P = 0.32, estradiol P = 0.08, progesterone P = 0.20, and testosterone P = 0.11, n = 7 pairs). Therefore, any duplicate samples from the same individual were averaged to give final concentrations for each hormone and allowed steroid hormone concentrations to be compared among all samples regardless of the skeletal bone analyzed. This is in agreement with Yarrow et al. (2010), where testosterone measured in tibias and femurs of rats (*Rattus* spp.) were similar.

Steroid hormone concentrations were not normally distributed; therefore, nonparametric permutational analysis of variance with a priori pairwise comparisons (PERMANOVAs) and Kruskal-Wallis Analysis of Variance (ANOVAs) were used. Steroid hormone concentrations were analyzed both as a group (all hormones analyzed via PERMANOVAs) and individually (progesterone, testosterone, cortisol, and estradiol concentrations via Kruskal-Wallis ANOVAs) to determine significant differences among archaeological, historical, and modern periods

(sample time periods), decades, and between sexes (Anderson 2001, Anderson and Walsh 2013, Wang et al. 2016). A Mann-Whitney pairwise comparison test was performed when the Kruskal-Wallis tests were significant. Bray-Curtis similarity matrices paired with nonmetric Multidimensional Scaling (nMDS) plots with 50 restarts were used to visually show similarities among samples based on steroid hormone concentrations. Discriminant function analysis (DFA) was used to determine if hormone concentrations of known sexed individuals could help determine the sex of unknown individuals. Similarity percentages (One-Way SIMPER) was used to determine which hormone concentrations contributed to differences among different time periods (Mejri et al. 2014). nMDS and SIMPER analyses were done in PRIMER (V6, Clarke and Gorley 2006). Correlations between steroid hormone concentrations and walrus population size throughout historical time periods (1830s – 2010s) were analyzed using Spearman's rank correlation (Wilson et al. 1997). PERMANOVAs, Kruskal-Wallis ANOVAs, and Spearman's rank correlations were performed in PAST (V 3.10, Hammer et al. 2001). The core software of R was used to create visual representations of data and run DFA of hormone concentrations (R Core Team 2013). An alpha of 0.05 was used for all analyses. All reported statistical differences are among the steroid hormone concentrations' medians, as medians are robust to outliers. All data are reported as median ± 1 SD, with mean values reported for reference.

3.4 Results

Non-detectable concentrations of steroid hormones in all walrus bone samples were as followed: cortisol ($n = 0$), estradiol ($n = 5$), progesterone ($n = 21$), and testosterone ($n = 0$).

3.4.1 Comparing Steroid Hormone Concentrations Between Sexes

Female walruses had significantly different steroid hormone concentrations compared with males (PERMANOVA, $P = 0.03$) and unknown sexed individuals ($P = 0.03$), when all samples were pooled across all time periods. Males and unknown sexes had similar hormone concentrations ($P = 0.35$). All steroid hormone concentrations were higher in females compared with males and unknown sexes (Figure 3.2 A-D). However, samples did not visually group together by sex based on similarities of hormone concentrations (nMDS, Figure 3.3) or by discriminant function analysis of hormone concentrations (DFA, 56 % matching success for unknown samples); therefore, assigning sex to unknown samples based on hormone concentrations was not possible in this study. Overlapping ranges in hormone concentrations among sexes and age classes adds to the difficulty in assigning sex to unknown individuals solely based on these four steroid hormone concentrations (Table 3.4).

3.4.2 Steroid Hormone Concentrations from Archaeological, Historical, and Modern Time Periods

Steroid hormone concentrations were significantly different among sample time periods (PERMANOVA, $P = 0.03$). Historical (200 − 20 BP) steroid hormone concentrations were significantly different from hormones in modern (2014 and 2015, PERMANOVA a priori pairwise test, $P = 0.04$) bones, but not from archaeological samples (> 200 BP, $P = 0.05$). Archaeological and modern samples had similar steroid hormone concentrations ($P = 0.31$, Table 3.5). Cortisol concentrations in historical samples were significantly higher compared with archaeological samples (Kruskal-Wallis ANOVA, $P = 0.008$), but not higher than modern samples ($P = 0.07$).

The contribution of each steroid hormone (SIMPER) to the differences among sample time periods is summarized in Figure 3.4. Overall, differences in reproductive hormones, specifically progesterone and estradiol, contributed the most to the differences observed among sample time periods. Cortisol contributed < 10 % to the differences among sample time periods.

Steroid hormone concentrations were highly variable among sample time periods. Based on similarities of hormone concentrations among sample time periods, separate clusters of only historical, archaeological, and modern samples are present (nMDS, Figure 3.4). However, at other points throughout sample time periods, samples from all time periods are clustered together, displaying the high variability of walrus bone steroid hormone concentrations through time (Figure 3.4). This high variability is due to differences in estradiol and progesterone concentrations (SIMPER, Figure 3.4).

3.4.3 Decadal Steroid Hormone Concentrations (1830s – 2010s)

Steroid hormone concentrations were not significantly different for all decades (PERMANOVA, $P = 0.11$). However, when incorporating sex, both sex and the interaction term between sex and decade were significant ($P = 0.04$ for both sex and sex*decade). Differences among decades can therefore be explained by the sex of the individuals sampled during each respective decade. When separating out walrus samples by sex (leaving out unknown individuals), only females had significantly different steroid hormone concentrations among decades ($P = 0.004$ and $P = 0.22$ for females and males, respectively). However, available samples were skewed towards males, lessening the power to detect significant changes in hormone concentrations in the entire population over time that were represented by males only. Female samples were only available for the 1930s, 1950s, 1960s, 1970s, and 2010s (Table 3.6).

When comparing individual steroid hormone concentrations separately among decades with all samples included, all steroid hormone concentrations were significantly different among decades (Kruskal Wallis-ANOVAs, Table 3.7 A-E**).** In general, the early historical decades (1830s – 1930s) were similar to the 2010s, but different from the 1950s – 1980s (Table 3.7 B-E). All steroid hormones followed a similar trend, where hormone concentrations were lower in the 1830s and persisted with similar concentrations until the 1930s, when hormone concentrations increased significantly (Table 3.7 B-E, Figure 3.5 A-D). All steroid hormone concentrations increased until reaching peak mean concentrations in the 1960s (Table 3.6). Mean hormone concentrations started to decrease in the 1970s, but not significantly across all steroid hormone concentrations until the 2010s (Table 3.7 B-E, Figure 3.5 A-D**).**

Focusing on decades with significantly higher steroid hormone concentrations compared with modern samples from the 2010s (Table 3.7 B-E), progesterone and estradiol contributed most to the dissimilarities among decades (SIMPER, Table 3.8). Cortisol contributed < 10 % to differences among decades. Samples from different decades can be grouped based on progesterone concentrations (Appendix 3.4). Samples from the 1950s – 1970s had high concentrations of progesterone, where samples from 2014 had significantly lower levels, and samples from the 1880s – 1920s, and 2015 were mostly male or of unknown sex (Table 3.6) and had the lowest progesterone concentrations (Figure 3.5 A, Table 3.6).

3.4.4 Steroid Hormone Concentrations and Minimum Walrus Population Estimates

Cortisol (r = -0.15, P = 0.03), progesterone (r = -0.21, P = 0.006), and testosterone (r = -0.29, P < 0.001) had significant negative correlations with the minimum population estimate of walruses, but estradiol concentrations did not significantly correlate with population size (P =

0.32, Table 3.9). This correlation was performed again with only females, because they have significantly higher and different hormone concentrations through time compared with males and unknown sex samples as described above. The correlation to the minimum population estimate improved for progesterone (r = -0.42, P = 0.002 females only) and estradiol (r = -0.36, P = 0.008 females only), but not testosterone (r = -0.25, P = 0.07 females only) or cortisol (r = -0.13, P = 0.13 females only; Table 3.9).

3.5 Discussion

3.5.1 Stress Response to Current Climate Change and Cortisol Concentrations

One of our main objectives was to address the stress response of the present-day walrus population to the current changing climate in the Arctic and the lack of a sea ice platform. Our results show that the current walrus population has similar bone cortisol concentrations (i.e., stress response) compared to archaeological and historical populations, indicating a physiological resilience to current Arctic conditions (Table 3.5). The archaeological samples are at a minimum 215 years old (Appendix 3.1). This is only 75 years after the start of the industrial revolution in 1750 (IPCC 2013), and the majority of our samples were radiocarbon dated before that time. While recent significant warming in the Arctic began in the 1950s (Kaufman et al. 2009), the Industrial Revolution is the start of anthropogenic emissions of CO_2, which has contributed to the rapid rate of climate change in the Arctic (IPCC 2013, Kinnard et al. 2011). Therefore, our archaeological dataset is as close to a "true" baseline walrus population as possible and provides a reasonable control in our study.

Modern samples are from the past two years and represent a population that could be expected to exhibit a stress response due to a reduction of summer sea ice over the last nine years

(Jay et al. 2012, MacCracken 2012). In 2015, up to 35000 walruses hauled out on the beach near Point Lay instead of utilizing sea ice haulouts, possibly a climate change response (Jay et al. 2012). Potential stressors related to lack of sea ice in the Chukchi Sea include longer foraging trips for calves and females that have high energy demands (Jay et al. 2012, Noren et al. 2014), decreases in calf survival due to human and natural-induced stampedes (Jay et al. 2012, Udevitz et al. 2013), depleted benthic food sources (Sheffield and Grebmeier 2009, Jay et al. 2011), and potentially increased encounters with polar bears (*Ursus maritimus*) spreading novel diseases to the population (Burek et al. 2008, Garlich-Miller et al. 2011). However, our results show that walruses do not have significantly increased cortisol concentrations over the past nine years compared with our control samples (i.e., archaeological bone), nor did cortisol concentrations contribute more than 10 % to the significant differences in hormone concentrations among decades and archaeological, historical, and modern time periods (Figure 3.4, Table 3.8).

Bone cortisol concentrations potentially represent an averaged hormone signature over the lifespan of a walrus (Table 3.3), however, the actual reservoir time of hormones in bones is unknown (Yarrow et al. 2010). The majority of the modern samples are adult animals based on tooth data and hunter age class information (Appendix 3.3). Therefore, the bulk of our modern samples are at a minimum 14 years old (Fay 1982). Assuming our estimated complete bone hormone turnover rate of ~33 years is accurate, an increased stress response, as a result of reduced sea ice abundance in the past nine years, should have manifested in bone cortisol concentrations. Walruses have persevered through previous warming and cooling periods in the Arctic and have lived through periods of contractions and extensions of sea ice (Dyke et al. 1999, Garlich-Miller et al. 2011). Genetically, there is still high diversity within the population

indicating a potential resiliency to past environmental changes (Sonsthagen et al. 2012). Therefore, it is not necessarily unexpected that the modern walrus population has similar cortisol concentrations compared to the archaeological population. This supports the idea that walruses have resiliency to the current receding sea ice.

Walruses exhibited a significantly higher stress response in the 1950s and 1960s compared with modern-day walruses (Table 3.7 D, Figure 3.5 C). A possible explanation for the elevated cortisol concentrations is the exponential increase of the walrus population during the 1950s – 1970s (Fay et al. 1989, 1997, Garlich-Miller et al. 2011). Highly fecund females would have higher cortisol concentrations (Gardiner and Hall 1997), because of the need for increased energy stores (Noren et al. 2014), carrying a fetus (Hunt et al. 2014), physically giving birth, caring for the calf after birth, and protecting their calf from predators and other dangers (Fay 1982). Reproductively active males would have higher cortisol concentrations (Bartsh et al. 1992) during this fecund period due to competition with other males for reproductive females (Fay 1982). Thus, the increased cortisol concentrations observed in walrus bone from the 1950s and 1960s compared with the 2010s is likely due to this reproductively active twenty-year time period. In addition to the population increasing in the 1950s and 1960s, competition for resources during this reproductive period could contribute to the increased cortisol concentrations. However, clam populations did not exhibit signs of depletion in walrus habitat until the 1970s (Lowry et al. 1980), and the stress response to this depletion of resources and subsequent increase in competition measured in bone would potentially not be detectable until the 1980s. Only two samples were available for analysis in the 1980s, however more samples are being collected and analyzed to fill in this and other time gaps.

In this study, cortisol concentrations did not increase in response to commercial walrussing during the 1850s – 1900s (Figure 3.5 C, Fay et al. 1989, Bockstoce 1995, Taylor and Udevitz 2015). Commercial whalers took at a minimum 150000 walruses with 85% of these kills occurring from 1869 – 1878 (Bockstoce 1995). The lack of an increased stress response during these decades was an expected result because; bone steroid hormones do not capture an acute stress response, such as pursuit by whalers, due to the slow turnover in bone. This is a limitation of bone steroid hormone studies; the potential stressor needs to be chronic and long-term to get a substantial amount of cortisol incorporated into the bone. We have to interpret our results from these decades (1850s – 1900s) with caution due to low sample sizes and only having males and unsexed individuals available (Table 3.6), but our data support the notion of bone steroid hormones being an effective monitor of chronic stressors (i.e., reduction of sea ice habitat), but not acute stressors (i.e., commercial hunting).

3.5.2 Reproductive Status of the Walrus Population

Another objective of this study was to assess bone reproductive hormones (i.e., estradiol, progesterone, and testosterone) as a long-term monitoring tool for reproductive status of the walrus population. As mentioned, Arctic sea ice is receding, and walruses are utilizing terrestrial haulouts in the summer (Jay et al. 2012) with predictions of a decrease in population carrying capacity (MacCracken 2012) and calf survival (Udevitz et al. 2013). Thus, an investigation into a new long-term monitoring tool for the reproductive status of the walrus population is warranted. Using the available minimum walrus population estimates from the literature, and a walrus specific geometric growth rate equation (Udevitz et al. 2013), we were able to approximate past populations from the 1830s – 2010s (Tables 3.1, 3.3). Progesterone and testosterone

concentrations were negatively correlated with the estimated minimum walrus population size (Table 3.9). Thus, when the walrus population is constant and/or high, possibly at carrying capacity, hormone concentrations are low, and when population numbers are low, hormone concentrations are relatively high. These significant correlations complement our decadal differences in steroid hormones. When the population is increasing substantially (1950s – 1970s), hormone concentrations in these decades are significantly higher compared with other decades, when the population is either decreasing or at carrying capacity (Figure 3.5 A, B, D). However, progesterone and estradiol should be the main hormones to analyze for long-term changes in reproductive status, because they contributed the most to differences among decades and archaeological, historical, and modern time periods (Figure 3.4, Table 3.8), and they are the main female reproductive hormones. In addition, when analyzing only females, both progesterone and estradiol had a negative correlation with the minimum walrus population estimate (Table 3.9).

As territorial breeders, walruses need fewer males than females to maintain a healthy population (Fay 1982, USFWS Stock Assessment 2014). The current walrus population has low progesterone and estradiol concentrations compared with decades of known population increases (1950s – 1970s, Figure 3.5 A, D, Table 3.6). The most recent walrus population estimate was in 2006, but it was a conservative estimate (mean = 129000 animals, 55000 - 507000 95 % CI), because of poor weather conditions inhibiting the survey of substantial portions of the walrus habitat (Speckman et al. 2011). The actual population most likely contains more animals than this estimate (Speckman et al. 2011, USFWS Stock Assessment 2014). If the population is around 250000 – 300000 animals (within the 95% confidence interval reported by Speckman et

al. 2011) the population would be at carrying capacity based on previous assessments (Fay et al. 1997, USFWS Stock Assessment 2014), although the current carrying capacity could be lower due to shifts in feeding locations and potentially lower benthic prey biomass (Grebmeier 2012, MacCracken 2012). Despite these uncertainties, progesterone and estradiol levels are low in the modern walrus population indicating possible low calf production and/or a population at carrying capacity.

Progesterone concentrations were highest in walrus bones from the 1960s, when the walrus population was increasing (Fay et al. 1989, 1997). This is expected, as progesterone is the main pregnancy hormone in walruses (Pomeroy 2011), and is elevated during pregnancy (Kellar et al. 2006, 2013, Muraco et al. 2012, Triggs 2013). Walruses have a longer gestation period (approximately 15 months; Fay 1982) than other pinnipeds (harbor seal (*Phoca vitulina*) and California sea lion (*Zalophus californianus*) at 11 months; Pomeroy 2011), therefore providing ample time for progesterone to be deposited into the bone. Calving intervals are estimated to be about one calf every 2 - 3 years (Fay 1982), meaning a fecund female could have constantly elevated progesterone concentrations throughout her reproductive life. While females had the highest progesterone concentrations, males also had relatively high progesterone concentrations (Table 3.4). This could be due to progesterone playing a facilitating role in male sexual behavior (Wagner 2006). Wagner (2006) suggested that during times of stress in male rats, circulating testosterone was inhibited and progesterone may play a supplementary role in facilitating male sexual behavior in response to the testosterone decrease. Another possibility for elevated progesterone concentrations in males is that progesterone is a precursor to testosterone, cortisol, and estradiol (Appendix 3.5, Koal et al. 2012). Progesterone may be stored in bones as a

"backup" hormone to metabolize other useful steroid hormones in case the animal cannot absorb or metabolize cholesterol into these important hormones (Simonen et al. 2000).

While bone progesterone concentrations are an effective tool for assessing the reproductive status of a population, they might be less reliable in sexing individuals or determining active pregnancy as has been shown for cetaceans (Appendix 3.4, Mansour et al. 2002, Kellar et al. 2006, 2013, Hunt et al. 2014). Progesterone had the highest number of non-detectable samples ($n = 21$), which could be attributed to having the lowest extraction efficiency (51 %) compared with the other hormones (cortisol = 72 %, estradiol = 79 %, and testosterone = 107 %). One way to improve on this extraction efficiency would be to extend the extraction time to 48 hours, compared with our 24-hour period, allowing more time for progesterone to be extracted from the walrus bone powder.

3.5.3 Estradiol

While estradiol was the hormone that contributed to the majority of differences among decades and sample time periods in our analysis (Figure 3.4, Table 3.8), it was not significantly correlated with the minimum walrus population estimate. Perhaps the lack of correlation is due to estradiol being an important component in maintaining bone mineral density in both males and females (Nguyen et al. 2014). In addition, estradiol can be locally synthesized in bone by the aromatization of testosterone (Yarrow et al. 2010), and our results from chapter 2 of this thesis supports the notion that walrus bone has similar endocrine capabilities. Thus, estradiol concentrations measured in walrus bone probably change seasonally or yearly (Chapter 2) unlike

the other steroid hormone concentrations measured in this study (Table 3.3). However, further work is needed to understand the role of estradiol in walrus bone health and reproductive status.

Even though estradiol appears to represent a relatively shorter accumulation time period in bone than our estimate (Table 3.3), estradiol concentrations followed similar trends seen in progesterone, cortisol, and testosterone during known times of an increasing walrus population (Figure 3.5 A-D). In addition, when analyzing females only, estradiol was significantly negatively correlated with population size (Table 3.9). Estradiol is an important hormone to prepare the female for pregnancy (Nelson 1990) and estrus (Robeck et al. 2005), and it influences female sexual behavior (Ogawa et al. 1998), which corresponds to estradiol concentrations only being significantly negatively correlated to the minimum population size for females, but not the entire sample set (both males and females) as seen for progesterone. Thus, estradiol may still be a useful hormone for monitoring reproductive status in bone, but probably only informative for females, as only female estradiol concentrations have a significant negative correlation with the minimum walrus population estimate (Table 3.9). Estradiol in bone had non-detectable samples ($n = 5$) of concentrations < 0.5 ng, with a relatively high extraction efficiency (our study = 79 %, Kellar et al. 2006 mean progesterone extraction efficiency of cetacean blubber = 71.1 %). This indicates estradiol concentrations, if non-detectable, are low in walrus bone and is not an artifact of our method.

3.5.4 Testosterone

Testosterone was significantly negatively correlated with walrus population size (Table 3.9), but contributed < 25 % to differences in steroid hormone concentrations among sample time

periods or decades (Figure 3.4, Table 3.8). Testosterone is the male reproductive hormone and may not be as essential for monitoring the reproductive status of the walrus population compared with the major female reproductive hormones, progesterone and estradiol. Male walruses are territorial breeders with a mean of one mature mating male for every 23 female mates (Fay 1982), thus fewer males are needed for sustaining a healthy population. While adult males had the highest testosterone concentrations (41718 ng/g lipid and 14392 ng/g lipid for male and female, respectively), females had higher median concentrations (892.68 ± 3036.67 ng/g lipid, 313.27 ± 5089.78 ng/g lipid for females and males, respectively). In humans, high salivary testosterone concentrations in females were associated with an increase in attractiveness to males (Welling et al. 2007). It is possible that the higher testosterone concentration in females in this study were driven by the high testosterone concentrations during the 1950s – 1970s, increasing female receptivity to males during that time period, therefore aiding in the population increase during those decades (Figure 3.2 B). In free-ranging hybrid baboons (*Papio* spp.), females who had higher dominance and/or were pregnant had higher testosterone concentrations than lower ranking and non-pregnant females (Beehner et al. 2005). In addition, elevated testosterone has been shown during gestation in female rats (Weisz and Ward 1980) and the reproductive cycle of female humans (Judd and Yen 1973). In this study, female walruses with high testosterone concentrations could be indicative of older, reproductively active animals in the walrus herd. These females with high testosterone could potentially be giving birth to a majority of males (Helle et al. 2008). This could be detrimental on a population level given more females are needed to sustain a healthy walrus population (Fay 1982), but might be a self-regulating mechanism on the population.

The testosterone extraction efficiency was 107 %, indicating cross reactivity with other similar metabolites (Yarrow et al. 2010); therefore, testosterone hormone levels might be biased toward higher concentrations. In addition, females may sequester testosterone to use as a direct precursor to estradiol (Appendix 3.5, Koal et al. 2012). Lastly, it is possible that some specimens were sexed incorrectly during collection. Further studies are needed to determine the biological relevance of elevated testosterone levels in female walruses, and its involvement in female reproductive status.

3.5.5 Conclusions

This is the first study to assess steroid hormone concentrations from archaeological, historical, and modern marine mammal bone as an effective tool to monitor chronic stress response and long-term changes in reproductive status. Currently, walruses have similar cortisol concentrations to archaeological and historical populations indicating a physiological resilience to changing climate and receding sea ice. The walrus population had higher cortisol concentrations in the past when hunting pressure actually decreased and the population increased rapidly. As more archaeological samples are processed and dated, finer archaeological periods can be analyzed to determine how walruses have responded to previous warming and cooling periods in the Arctic. This will further our understanding of physiological resilience of walruses to past periods of change. It is well known that progesterone and testosterone concentrations can determine reproductive status of an individual (Kellar et al. 2006, 2013, Pérez et al. 2011, Trego et al. 2013, Zhang et al. 2014), and our results complement these studies by validating bone reproductive hormones, progesterone and testosterone, as valuable tools for monitoring long-term reproductive status of a population. In addition, our study validated estradiol from walrus

bone as a tool for long-term monitoring of changes in the reproductive status of females from the walrus population. However, the local production of estradiol could skew estradiol to a possibly faster turnover rate and shorter reservoir time in cortical bone, which warrants further investigation. Differences in estradiol and progesterone contributed the most to the dissimilarities among walrus bone steroid hormone concentrations through time compared to testosterone. Thus, estradiol and progesterone are suitable reproductive hormones for future walrus studies monitoring the reproductive status of the population using bone. The current walrus population has low progesterone and estradiol concentrations that are similar to hormone concentrations in archeological samples possibly indicating low calf production and a population that is approaching carrying capacity. An updated walrus population estimate is urgently needed to further interpret current steroid hormone trends.

Our novel method of using bone steroid hormones is pertinent for management and conservation of walruses, Arctic marine mammals in general, and other threatened or endangered species. The results from this study will help management and co-management groups decide if walruses should be listed under the Endangered Species Act by the end of 2017 (USFWS 2014). Our results show the modern walrus population exhibited a similar stress response (i.e., cortisol concentrations) compared with archaeological (i.e., baseline stress response) and historical walrus populations. However, with conditions in the Arctic predicted to worsen, and the walrus summer sea ice habitat expected to be gone as early as 2050 (IPCC 2013), as well as other stressors on the rise (e.g., ship traffic, chemical and noise pollution) continued monitoring of walrus cortisol concentrations in the coming years is warranted. Our research could be extremely useful for assessing physiological responses to climate change in other pagophilic marine

mammals, including polar bears and ice seals. Museum collections, such as UAM and Smithsonian Institution National Museum of Natural History and other archived sample databases (e.g., stranding networks), could potentially house specimens to conduct similar bone steroid hormone studies on various marine mammal species.

3.6 Acknowledgements

The authors thank the Native hunters from the St. Lawrence Island communities of Savoonga and Gambell, who provided the majority of the bone samples from 2014 – 2015, without their collaboration this project could not have been done. Thank-you to our collaborators: Alaska Department of Fish and Game, Eskimo Walrus Commission, hunters from Barrow, North Slope Borough Department of Wildlife Management, Purdue University, Smithsonian Institution National Museum of Natural History, University of Alaska Museum, and U.S. Fish and Wildlife Service. The catalog numbers of specimens used from both UAM and the Smithsonian Institute National Museum of Natural History are listed in the Appendices. A special thank-you to Dr. M. Wooller who provided helpful comments and edits to this manuscript. Thank-you to C. Clark, A. Jannasch at Purdue University, and the undergraduate technicians, S. George, C. Markel, E. VanDam, and L. Wendling, who provided support throughout this project. This project is based on work supported by the National Science Foundation under Grant No. 1263848 with contributions from BOEM. Additional funding was provided by a UAF Center for Global Change Student Research Grant with funds from the Cooperative Institute for Alaska Research and the Dr. Donald Hood Memorial Scholarship for Marine Science.

3.7 References

Accorsi, P. A., E. Carloni, P. Valsecchi, R. Viggiani, M. Gamberoni, C. Tamanini, and E. Seren. 2008. Cortisol determination in hair and faeces from domestic cats and dogs. General and Comparative Endocrinology 155:398–402.

Anderson, M. J. 2001. A new method for non-parametric multivariate analysis of variance. Austral Ecology 26:32–46.

Anderson, M. J., and D. C. I. Walsh. 2013. PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: What null hypothesis are you testing? Ecological Monographs 83:557–574.

Atkinson, S., C. Combelles, D. Vincent, P. Nachtigall, J. Pawloski, and M. Breese. 1999. Monitoring of progesterone in captive female false killer whales, *Pseudorca crassidens*. General and Comparative Endocrinology 115:323–332.

Bartsh, S. S., S. D. Johnston, and D. B. Siniff. 1992. Territorial behavior and breeding frequency of male Weddell seals (*Leptonychotes weddelli*) in relation to age, size, and concentrations of serum testosterone and cortisol. Canadian Journal of Zoology 70:680–692.

Beehner, J. C., J. E. Phillips-Conroy, and P. L. Whitten. 2005. Female testosterone, dominance rank, and aggression in an Ethiopian population of hybrid baboons. American Journal of Primatology 67:101–119.

Bockstoce, J. R. 1995. Whales, Ice, and Men: The History of Whaling in the Western Arctic, 2nd ed. University of Washington Press, Seattle and London, Washington.

Bowman, S. 1990. Radiocarbon Dating. University of California Press/British Museum, Los Angeles, California, USA.

Bryan, H. M., A. G. Adams, R. M. Invik, K. E. Wynne-Edwards, and J. E. G. Smits. 2013. Hair as a meaningful measure of baseline cortisol levels over time in dogs. Journal of the American Association for Laboratory Animal Science 52:189–96.

Burek, K. A., F. M. D. Gulland, and T. M. O'Hara. 2008. Effects of climate change on Arctic marine mammal health. Ecological Applications 18:126–134.

Caldwell, S. 2013. Disaster declared for subsistence walrus hunt on St. Lawrence Island. Alaska Dispatch News. Retrieved from http://www.adn.com/article/20130902/disaster-declared-subsistence-walrus-hunt-st-lawrence-island (accessed December 11, 2015)

Cherkinsky, A. 2009. Can we get a good radiocarbon age from "bad bone"? Determining the reliability of radiocarbon age from bioapatite. Radiocarbon 51:647–655.

Clarke, K. R., and R. N. Gorley. 2006. PRIMER v6: User manual/tutorial. PRIMER-E, Plymouth.

Clarke, B. 2008. Normal bone anatomy and physiology. Clinical Journal of the American Society of Nephrology 3:131–139.

Collins, M. J., C. M. Nielsen-Marsh, J. Hiller, C. I. Smith, J. P. Roberts, R. V. Prigodich, T. J. Wess, J. Csapò, A. R. Millard, and G. Turner-Walker. 2002. The survival of organic matter in bone: A review. Archaeometry 3:383–394.

Cooper, L. W., C. J. Ashjian, S. L. Smith, L. A. Codispoti, J. M. Grebmeier, R. G. Campbell, and E. B. Sherr. 2006. Rapid seasonal sea-ice retreat in the Arctic could be affecting Pacific walrus (*Odobenus rosmarus divergens*) recruitment. Aquatic Mammals 32:98–102.

Cronin, T. M., and M. A. Cronin. 2015. Biological response to climate change in the Arctic Ocean: the view from the past. Arktos 1:1–18.

Davis, S. 1992. A rapid method for recording information about mammal bones from archaeological sites. Ancient Monuments Laboratory Report 19:1–16.

Dehn, L. A., G. G. Sheffield, E. H. Follmann, L. K. Duffy, D. L. Thomas, G. R. Bratton, R. J. Taylor, and T. M. O'Hara. 2005. Trace elements in tissues of phocid seals harvested in the Alaskan and Canadian Arctic: influence of age and feeding ecology. Canadian Journal of Zoology 83:726–746.

Difrancesco, R., V. Frerichs, J. Donnelly, C. Hagler, J. Hochreiter, and K. M. Tornatore. 2007. Simultaneous determination of cortisol, dexamethasone, methylprednisolone, prednisone, prednisolone, mycophenolic acid and mycophenolic acid glucuronide in human plasma utilizing liquid chromatography-tandem mass spectrometry. Journal of Chromatography B 859:42–51.

Dumond, D. E., and D. G. Griffin. 2002. Measurements of the marine reservoir effect on radiocarbon ages in the eastern Bering Sea. Arctic 55:77–86.

During, A., G. Penel, and P. Hardouin. 2015. Understanding the local actions of lipids in bone physiology. Progress in Lipid Research 59:126–146.

Dyke, A. S., J. Hooper, C. R. Harington, and J. M. Savelle. 1999. The late Wisconsinan and Holocene record of walrus (*Odobenus rosmarus*) from North America: A review with new data from Arctic and Atlantic Canada. Arctic Institute of North America 52:160–181.

Evershed, R. P., G. Turner-Walker, R. E. M. Hedges, N. Tuross, and A. Leyden. 1995. Preliminary results for the analysis of lipids in ancient bone. Journal of Archaeological Science 22:277–290.

Fair, P. A., and P. R. Becker. 2000. Review of stress in marine mammals. Journal of Aquatic Ecosystem Stress and Recovery 7:335–354.

Fair, P. A., A. M. Schaefer, T. A. Romano, G. D. Bossart, S. V. Lamb, and J. S. Reif. 2014. Stress response of wild bottlenose dolphins (*Tursiops truncatus*) during capture-release health assessment studies. General and Comparative Endocrinology 206:203–212.

Fay, F. H. 1957. History and present status of the Pacific walrus population. Transactions of the North American Wildlife Conference 22: 431–445.

Fay, F. H. 1982. Ecology and biology of the Pacific walrus, *Odobenus rosmarus divergens* Illiger. North American Fauna 74:1–266.

Fay, F. H., and J. J. Burns. 1988. Maximal feeding depth of walruses. Arctic 41:239–240.

Fay, F. H., B. P. Kelly, and J. L. Sease. 1989. Managing the exploitation of Pacific walruses: a tragedy of delayed response and poor communication. Marine Mammal Science 5:1–16.

Fay, F. H., L. L. Eberhardt, B. P. Kelly, J. J. Burns, and L. T. Quakenbush. 1997. Status of the Pacific walrus population, 1950-1989. Marine Mammal Science 13:537–565.

Fidel, M., A. Kliskey, L. Alessa, and O. P. Sutton. 2014. Walrus harvest locations reflect adaptation: a contribution from a community-based observation network in the Bering Sea. Polar Geography 37:48–68.

Gardiner, K. J., and A. J. Hall. 1997. Diel and annual variation in plasma cortisol concentrations among wild and captive harbor seals (*Phoca vitulina*). Canadian Journal of Zoology 75:1773–1780.

Garlich-Miller, J. L., L. T. Quakenbush, and J. F. Bromaghin. 2006. Trends in age structure and productivity of Pacific walruses harvested in the Bering Strait region of Alaska, 1952-2002. Marine Mammal Science 22:880–896.

Garlich-Miller, J. L., J. G. MacCracken, J. Snyder, R. Meehan, M. Myers, J. M. Wilder, E. Lance, and A. Matz. 2011. Status review of the Pacific walrus (*Odobenus rosmarus divergens*). U.S. Fish and Wildlife Service, Marine Mammals Management, Anchorage, AK.

George, J. C., and J. R. Bockstoce. 2008. Two historical weapon fragments as an aid to estimating the longevity and movements of bowhead whales. Polar Biology 31:751–754.

Gilbert, R. O. 1987. Statistical methods for environmental pollution monitoring. John Wiley and Sons, Inc., New York, NY, USA.

Gilbert, J. R., G. A. Fedoseev, D. Seagars, E. Razlivalov, and A. LaChugin. 1992. Aerial census of Pacific walrus, 1990. US Fish and Wildlife Service Marine Mammals Management Technical Report 91–1, Anchorage, AK.

Grayson, D. K. 1978. Minimum numbers and sample size in vertebrate faunal analysis. Society for American Archaeology 43:53–65.

Grayson, D. K. 1984. Quantitative zooarchaeology: topics in the analysis of archaeological faunas. Academic Press, Inc., Orlando, Florida, USA.

Grebmeier, J. M., L. W. Cooper, H. M. Feder, and B. I. Sirenko. 2006. Ecosystem dynamics of the Pacific-influenced northern Bering and Chukchi Seas in the Amerasian Arctic. Progress in Oceanography 71:331–361.

Grebmeier, J. M. 2012. Shifting patterns of life in the pacific Arctic and sub-Arctic Seas. Annual Review of Marine Science 4:63–78.

Gulland, F. M. D., M. Haulena, and B. Hilliard. 1999. Adrenal function in wild and rehabilitated Pacific harbor seals (*Phoca vitulina richardsii*). Marine Mammal Science 15:810–817.

Hammer, Ø., D. A. T. Harper, and P. D. Ryan. 2001. Paleontological statistics software package for education and data analysis. Palaeontologia Electronica 4:9–18.

Harcourt, R. G., E. Turner, A. Hall, J. R. Waas, and M. Hindell. 2010. Effects of capture stress on free-ranging, reproductively active male Weddell seals. Journal of Comparative Physiology A 196:147–154.

Helle, S., T. Laaksonen, A. Adamsson, J. Paranko, and O. Huitu. 2008. Female field voles with high testosterone and glucose levels produce male-biased litters. Animal Behaviour 75:1031–1039.

Hogg, C. J., T. L. Rogers, A. Shorter, K. Barton, P. J. O. Miller, and D. Nowacek. 2009. Determination of steroid hormones in whale blow: It is possible. Marine Mammal Science 25:605–618.

Hufford, G., and T. Loughlin. 2009. Bering Sea: marine mammals in a regional sea. Pages 172–204 *in* G. Carleston Ray and J. McCormick-Ray, editors. Coastal-Marine Conservation: Science and Policy. Blackwell Science Ltd, Victoria, Australia.

Hunt, K. E., R. Stimmelmayr, C. George, C. Hanns, R. Suydam, H. Brower Jr, and R. M. Rolland. 2014. Baleen hormones: a novel tool for retrospective assessment of stress and reproduction in bowhead whales (*Balaena mysticetus*). Conservation Physiology 2:1–12.

IPCC. 2013. Climate Change 2013: The physical science basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. T. F. Stocker, D. Qin, G. K. Plattner, M. Tignor, S. K. Allen, J. Boschung, K. Nauels, Y. Xia, V. Bex, and P. M. Midgley, editors. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Jay, C. V., and Fischbach A. S. 2008. Pacific walrus response to Arctic Sea ice losses. U.S. Geological Survey, Fact Sheet 2008–3041. 4 pp.

Jay, C. V., B. G. Marcot, and D. C. Douglas. 2011. Projected status of the Pacific walrus (*Odobenus rosmarus divergens*) in the twenty-first century. Polar Biology 34:1065–1084.

Jay, C. V., A. S. Fischbach, and A. A. Kochnev. 2012. Walrus areas of use in the Chukchi Sea during sparse sea ice cover. Marine Ecology Progress Series 468:1–13.

Jay, C. V., J. M. Grebmeier, A. S. Fischbach, T. L. McDonald, L. W. Cooper, and F. Hornsby. 2014. Pacific walrus (*Odobenus rosmarus divergens*) resource selection in the Northern Bering Sea. PloS one 9:e93035.

Judd, H. L., and S. S. C. Yen. 1973. Serum androstenedione and testosterone levels during the menstrual cycle. The Journal of Clinical Endocrinology & Metabolism 36:475–481.

Kaufman, D. S., D. P. Schneider, N. P. Mckay, C. M. Ammann, R. S. Bradley, K. R. Briffa, G. H. Miller, B. L. Otto-Bliesner, J. T. Overpeck, B. M. Vinther, A. Lakes, and P. Members. 2009. Recent warming reverses long-term Arctic cooling. Science 325:4–7.

Kellar, N. M., M. L. Trego, C. I. Marks, and A. E. Dizon. 2006. Determining pregnancy from blubber in three species of delphinids. Marine Mammal Science 22:1–16.

Kellar, N. M., J. Keliher, M. L. Trego, K. N. Catelani, C. Hanns, J. C. George, and C. Rosa. 2013. Variation of bowhead whale progesterone concentrations across demographic groups and sample matrices. Endangered Species Research 22:61–72.

Kinnard, C., C. M. Zdanowicz, D. A. Fisher, E. Isaksson, A. De Vernal, and L. G. Thompson. 2011. Reconstructed changes in Arctic sea ice over the past 1,450 years. Nature 479:509–512.

Kjeld, M. 2001. Concentrations of electrolytes, hormones, and other constituents in fresh postmortem blood and urine of fin whales (*Balaenoptera physalus*). Canadian Journal of Zoology 79:438–446.

Koal, T., D. Schmiederer, H. Pham-Tuan, C. Röhring, and M. Rauh. 2012. Standardized LC-MS/MS based steroid hormone profile-analysis. Journal of Steroid Biochemistry and Molecular Biology 129:129–138.

Laidre, K. L., I. Stirling, L. F. Lowry, O. Wiig, M. P. Heide-Jørgensen, and S. H. Ferguson. 2008. Quantifying the sensitivity of Arctic marine mammals to climate-induced habitat change. Ecological. Applications 18:S97–S125.

Lowry, L. F., K. J. Frost, and J. J. Burns. 1980. Feeding of bearded seals in the Bering and Chukchi Seas and trophic interaction with Pacific walruses. Arctic 33:330–342.

Lowry, L. F., and F. H. Fay. 1984. Seal eating by walruses in the Bering and Chukchi Seas. Polar Biology 3:11–18.

MacCracken, J. G. 2012. Pacific walrus and climate change: observations and predictions. Ecology and Evolution 2:2072–2090.

MacCracken, J. G., and R. B. Benter. 2016. Trend in Pacific walrus (*Odobenus rosmarus divergens*) tusk asymmetry, 1990-2014. Marine Mammal Science 32:588–601.

Mansour, A. A. H., D. W. Mkay, J. Lien, J. C. Orr, J. H. Banoub, N. Øien, and G. Stenson. 2002. Determination of pregnancy status from blubber samples in minke whales (*Balaenoptera acutorostrata*). Marine Mammal Science 18:112–120.

Mark, L., Z. Patonai, A. Vaczy, N. Kajsza, and A. Marcsik. 2011. Hormone mass fingerprinting: novel molecular sex determination of ancient human skeletal remains. Pages 411–415 *in* I. Turbanti-Memmi, editor. Proceedings of the 37th International Symposium on Archaeometry; 12th-16th May, 2008, Siena, Italy. Springer-Verlag, Berlin, Heidelberg.

Mejri, S., C. Audet, G. W. Vandenberg, C. C. Parrish, and R. Tremblay. 2014. Biochemical egg quality in a captive walleye (*Sander vitreus*) broodstock population relative to ovulation timing following hormonal treatment. Aquaculture 431:99–106.

Metcalf, V., and M. Robards. 2008. Sustaining a healthy human-walrus relationship in a dynamic environment: challenges for comanagement. Ecological Applications 18:148–156.

Misarti, N., B. Finney, H. Maschner, and M. J. Wooller. 2009. Changes in northeast Pacific marine ecosystems over the last 4500 years: evidence from stable isotope analysis of bone collagen from archeological middens. The Holocene 19:1139–1151.

Muraco, H. S., L. D. Coombs, D. G. Procter, P. J. Turek, and M. J. Muraco. 2012. Use of human chorionic gonadotropin in a male Pacific walrus (*Odobenus rosmarus divergens*) to induce rut and achieve a pregnancy in a nulliparous female. Journal of Andrology 33:789–97.

Murtagh, R., V. Behringer, and T. Deschner. 2013. LC-MS as a method for non-invasive measurement of steroid hormones and their metabolites in urine and faeces of animals. Veterinary Medicine Austria 100:247–254.

Nelson, R. J., L. L. Badura, and B. D. Goldman. 1990. Mechanisms of seasonal cycles of behavior. Annual Review of Psychology 41:81–108.

Nguyen, H. T. T., B. Von Schoultz, T. V Nguyen, T. X. Thang, T. T. Chau, P. T. M. Duc, and A. L. Hirschberg. 2014. Sex hormone levels as determinants of bone mineral density and osteoporosis in Vietnamese women and men. Journal of Bone and Mineral Metabolism 33:658–665.

Noren, S. R., M. S. Udevitz, and C. V. Jay. 2014. Energy demands for maintenance, growth, pregnancy, and lactation of female Pacific walruses (*Odobenus rosmarus divergens*). Physiological and Biochemical Zoology 87:837–54.

Norris, D. 1997. Vertebrate Endocrinology, 3rd ed. Academic Press, San Diego, California.

Ogawa, S., V. Eng, J. Taylor, D. B. Lubahn, K. S. Korach, and D. W. Pfaff. 1998. Roles of estrogen receptor-α gene expression in reproduction-related behaviors in female mice. Endocrinology 139:5070–5081.

Pérez, S., Á. García-López, R. Stephanis, J. Giménez, S. García-Tiscar, P. Verborgh, J. M. Mancera, and G. Martínez-Rodriguez. 2011. Use of blubber levels of progesterone to determine pregnancy in free-ranging live cetaceans. Marine Biology 158:1677–1680.

Pomeroy, P. 2011. Reproductive cycles of marine mammals. Animal Reproduction Science 124:184–193.

R Core Team. 2013. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/.

Robeck, T. R., K. J. Steinman, M. Yoshioka, E. Jensen, J. K. O'Brien, E. Katsumata, C. Gili, J. F. McBain, J. Sweeney, and S. L. Monfort. 2005. Estrous cycle characterisation and artificial insemination using frozen-thawed spermatozoa in the bottlenose dolphin (*Tursiops truncatus*). Reproduction 129:659–674.

Rolland, R. M., K. E. Hunt, S. D. Kraus, and S. K. Wasser. 2005. Assessing reproductive status of right whales (*Eubalaena glacialis*) using fecal hormone metabolites. General and Comparative Endocrinology 142:308–317.

Schlechtriem, C., Focken, U., and K. Becker. 2003. Effect of different lipid extraction methods on δ13C of lipid and lipid-free fractions of fish and different fish feeds. Isotopes in Environmental and Health Studies 39:135-140.

Schonberg, S. V., J. T. Clarke, and K. H. Dunton. 2014. Distribution, abundance, biomass and diversity of benthic infauna in the Northeast Chukchi Sea, Alaska: Relation to environmental variables and marine mammals. Deep-Sea Research Part II: Topical Studies in Oceanography 102:144–163.

Seymour, J., L. Horstmann-Dehn, and M. J. Wooller. 2014. Proportion of higher trophic-level prey in the diet of Pacific walruses (*Odobenus rosmarus divergens*). Polar Biology 37:941–952.

Sheffield, G., and J. M. Grebmeier. 2009. Pacific walrus (*Odobenus rosmarus divergens*): differential prey digestion and diet. Marine Mammal Science 25:761–777.

Simonen, P., H. Gylling, A. N. Howard, and T. A. Miettinen. 2000. Introducing a new component of the metabolic syndrome: low cholesterol absorption. American Journal of Clinical Nutrition 72:82–88.

Sonsthagen, S. A., C. V. Jay, A. S. Fischbach, G. K. Sage, and S. L. Talbot. 2012. Spatial genetic structure and asymmetrical gene flow within the Pacific walrus. Journal of Mammalogy 93:1512–1524.

Speckman, S. G., V. I. Chernook, D. M. Burn, M. S. Udevitz, A. A. Kochnev, A. Vasilev, C. V. Jay, A. Lisovsky, A. S. Fischbach, and R. B. Benter. 2011. Results and evaluation of a survey to estimate Pacific walrus population size, 2006. Marine Mammal Science 27:514–553.

Spielhagen, R., K. Werner, S. Sørensen, K. Zamelczyk, E. Kandiano, G. Budeus, K. Husum, T. Marchitto, and M. Hald. 2011. Enhanced modern heat transfer to the Arctic by warm Atlantic water. Science 331:450–453.

Stuiver, M., P.J. Reimer, and R.W. Reimer. 2005. CALIB 5.0. [WWW program and documentation].

Taylor, R. L., and M. S. Udevitz. 2015. Demography of the Pacific walrus (*Odobenus rosmarus divergens*): 1974-2006. Marine Mammal Science 31:231–254.

Thomson, C., and J. Geraci. 1986. Cortisol, aldosterone, and leucocytes in the stress response of bottlenose dolphins, *Tursiops truncatus*. Canadian Journal of Fisheries and Aquatic Sciences 43:1010–1016.

Trego, M. L., N. M. Kellar, and K. Danil. 2013. Validation of blubber progesterone concentrations for pregnancy determination in three dolphin species and a porpoise. PLoS One 8:e69709.

Triggs, L. 2013. Establishing endocrine and behavioral parameters of reproduction in Pacific walrus. Thesis. University of Washington, Seattle, WA.

Trumble, S. J., E. M. Robinson, M. Berman-Kowalewski, C. W. Potter, and S. Usenko. 2013. Blue whale earplug reveals lifetime contaminant exposure and hormone profiles. Proceedings of the National Academy of Sciences of the United States of America 110:16922–16926.

Udevitz, M. S., R. L. Taylor, J. L. Garlich-Miller, L. T. Quakenbush, and J. A. Snyder. 2013. Potential population-level effects of increased haulout-related mortality of Pacific walrus calves. Polar Biology 36:291–298.

United States Fish and Wildlife Service (USFWS). 2014. Pacific walrus (*Odobenus rosmarus divergens*): Alaska Stock. United States Fish and Wildlife Service, Marine Mammals Management, Anchorage, AK. Available at http://www.fws.gov/alaska/fisheries/mmm/stock/stock.htm (accessed October 19, 2015)

United States Fish and Wildlife Service (USFWS). 2014. Pacific walrus: The Endangered Species Act and Alaska Native Hunting. United States Fish and Wildlife Service, Marine Mammals Management, Anchorage, AK. Available at http://www.fws.gov/alaska/fisheries/mmm/walrus/esa.htm (accessed March 11, 2016)

Wagner, C. K. 2006. The many faces of progesterone: A role in adult and developing male brain. Frontiers in Neuroendocrinology 27:340–359.

Wang, S. W., A. M. Springer, S. M. Budge, L. Horstmann, L. T. Quakenbush, and M. J. Wooller. 2016. Carbon sources and trophic relationships of ice seals during recent environmental shifts in the Bering Sea. Ecological Applications 26:830–845.

Weisz, J., and I. L. Ward. 1980. Plasma testosterone and progesterone titers of pregnant rats, their male and female fetuses, and neonatal offspring. Endocrinology 106:306–316.

Welling, L. L. M., B. C. Jones, L. M. DeBruine, C. A. Conway, M. J. Law Smith, A. C. Little, D. R. Feinberg, M. A. Sharp, and E. A. S. Al-Dujaili. 2007. Raised salivary testosterone in women is associated with increased attraction to masculine faces. Hormones and Behavior 52:156–161.

Wilson, B., P. Thompson, and P. Hammond. 1997. Habitat use by bottlenose dolphins: seasonal distribution and stratified movement patterns in the Moray Firth, Scotland. Journal of Applied Ecology 34:1365–1374.

Yarrow, J. F., C. F. Conover, J. A. Lipinska, C. A. Santillana, T. J. Wronski, and S. E. Borst. 2010. Methods to quantify sex steroid hormones in bone: applications to the study of androgen ablation and administration. American Journal of Physiology - Endocrinology and Metabolism 299:E841–E847.

Zhang, F., M. Bartels, D. Geter, M. Carr, L. McMlymount, T. Marino, and G. Klecka. 2009. Simultaneous quantitation of testosterone, estradiol, ethinyl estradiol, and 11-ketotestosterone in fathead minnow fish plasma by liquid chromatography/positive atmospheric pressure photoionization tandem mass spectrometry. Rapid Communications in Mass Spectrometry 23:3637–3646.

Zhang, P., Y. Yang, J. Han, Z. Lu, L. Wang, J. Tian, and Q. Wang. 2014. Serum testosterone, progesterone, and estradiol concentrations and sexual maturation in spotted seals (*Phoca largha*). Theriogenology 82:475–480.e4.

3.8 Figures

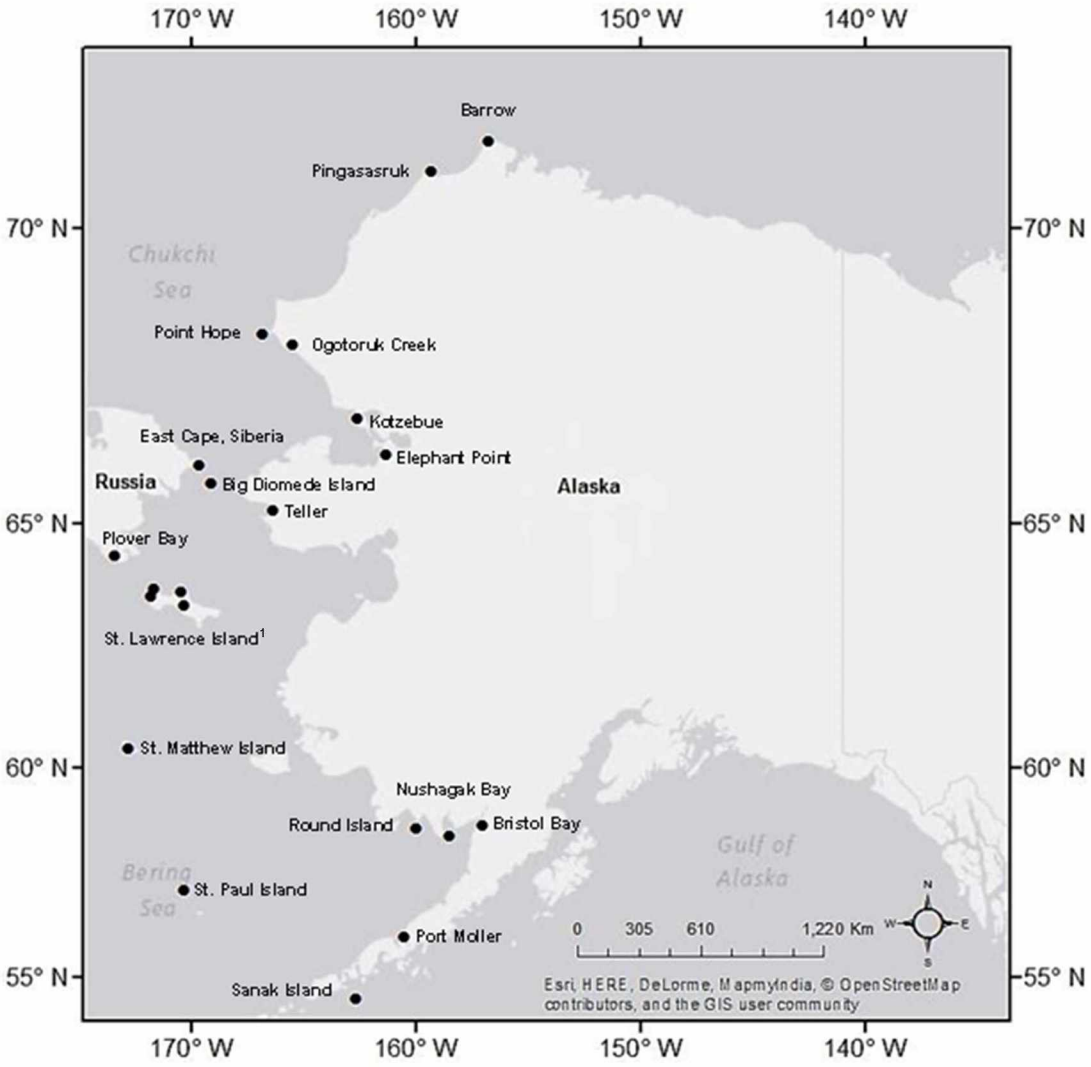


Figure 3.1: **Sampling locations of archaeological, historical, and modern Pacific walrus bone from Alaska and Russia.** Subscript "1" for St. Lawrence Island includes (from left to right) Kitnigipaluk, Gambell, Savoonga, and general St. Lawrence Island location.

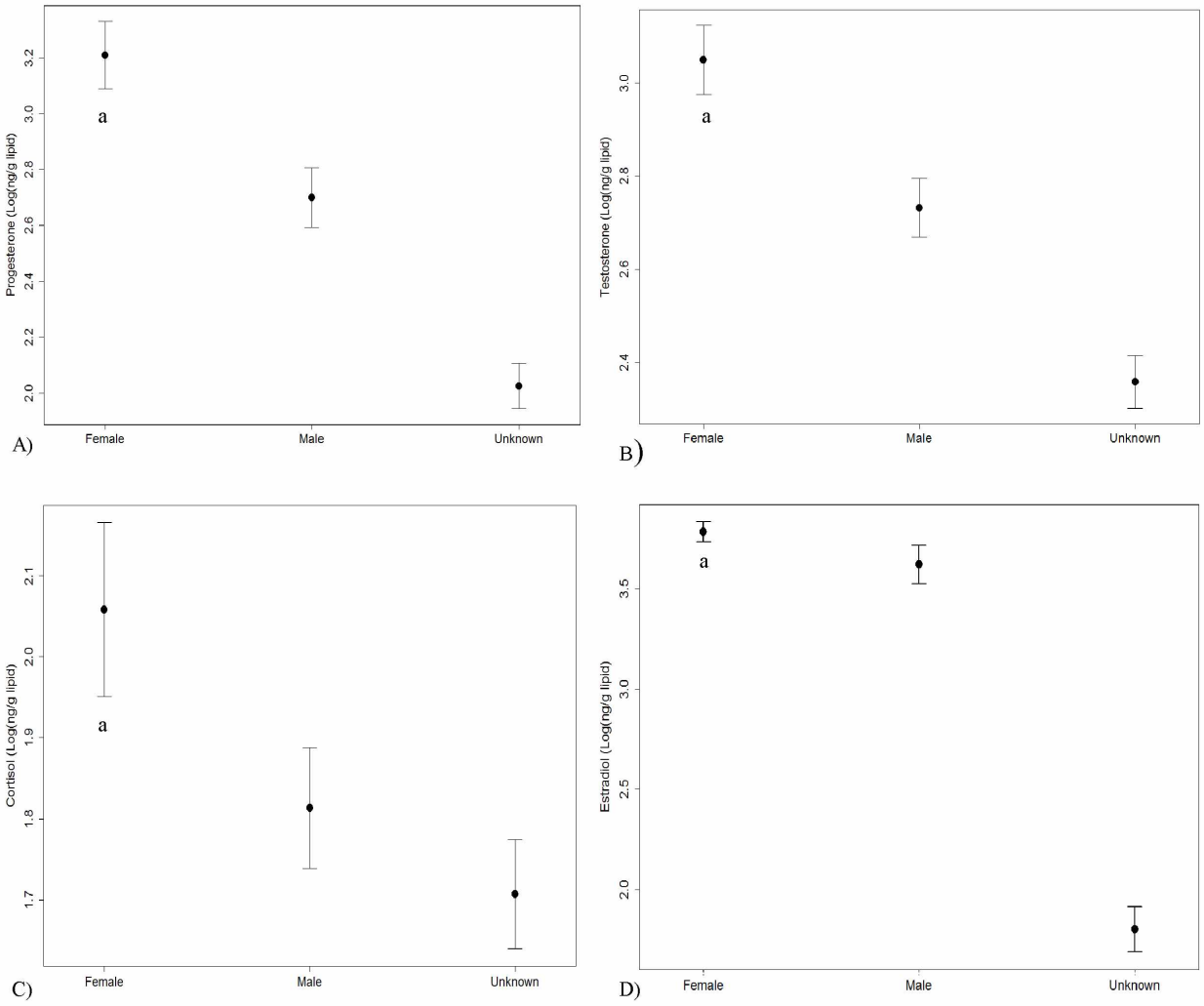


Figure 3.2 A-D: **Steroid hormone concentrations from all time periods plotted by sex.** Log transformed median ± 1 standard error (SE) steroid hormone concentrations of all walrus bone samples plotted by sex for progesterone (A), testosterone (B), cortisol (C), and estradiol (D). "a" indicates female walruses had significantly higher steroid hormone concentrations compared with unknown sexed and male walruses (PERMANOVA, $P = < 0.05$).

Comparison	Average Dissimilartiy	Percent (%) Contribution *Progesterone	Testosterone	Cortisol	*Estradiol
Female and Male	58	42	14	7	37
Female and Unknown	74	32	13	6	49
Male and Unknown	70	28	15	6	51

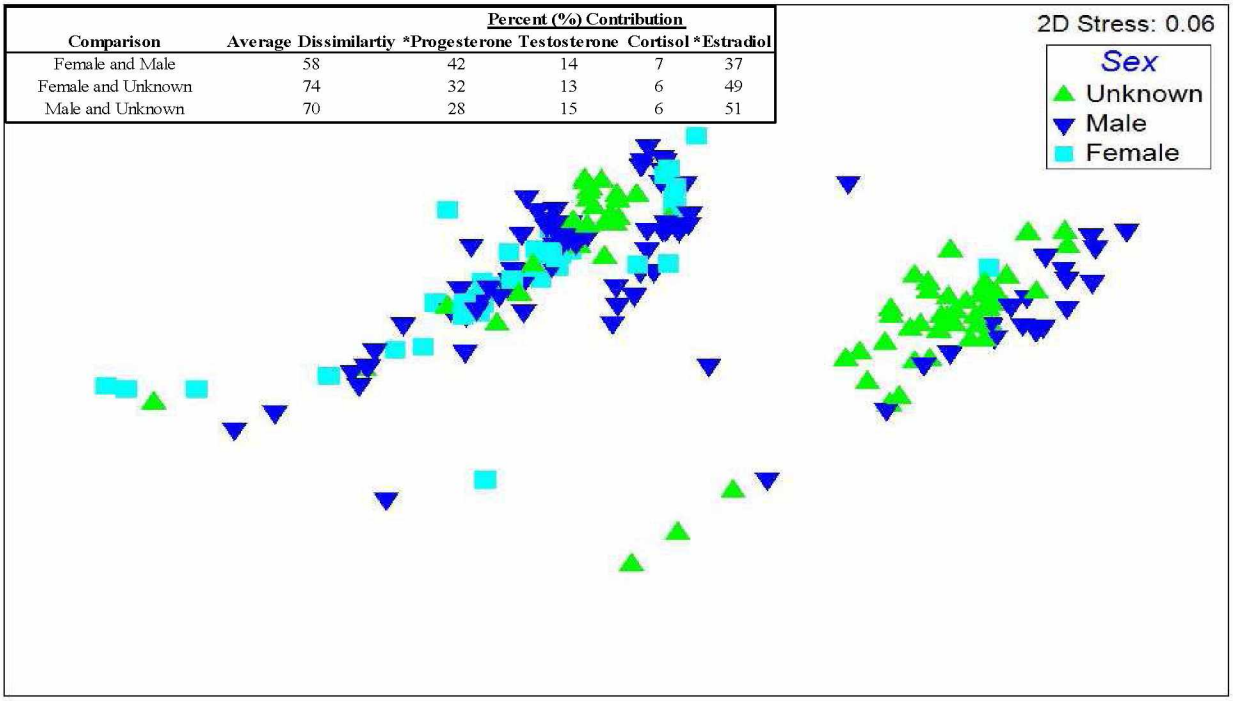


Figure 3.3: **Nonmetric dimensional scaling plot of all walrus samples based on similarities among all steroid hormone concentrations by sex with SIMPER results.** Both sexes and unknown sex samples are represented in both groups without obvious separation. An inset of the results from a One-Way SIMPER analysis shows which differences among hormone concentration contributed to the average dissimilarity between sexes. "*" notes estradiol and progesterone concentration differences among samples contributed the majority to the variability shown in the nMDS plot between sexes.

Comparison	Average Dissimilartiy	Percent (%) Contribution *Progesterone	Testosterone	Cortisol	*Estradiol
Archaeological and Historical	68	31	15	5	49
Archaeological and Modern	61	18	10	7	65
Modern and Historical	70	30	15	6	49

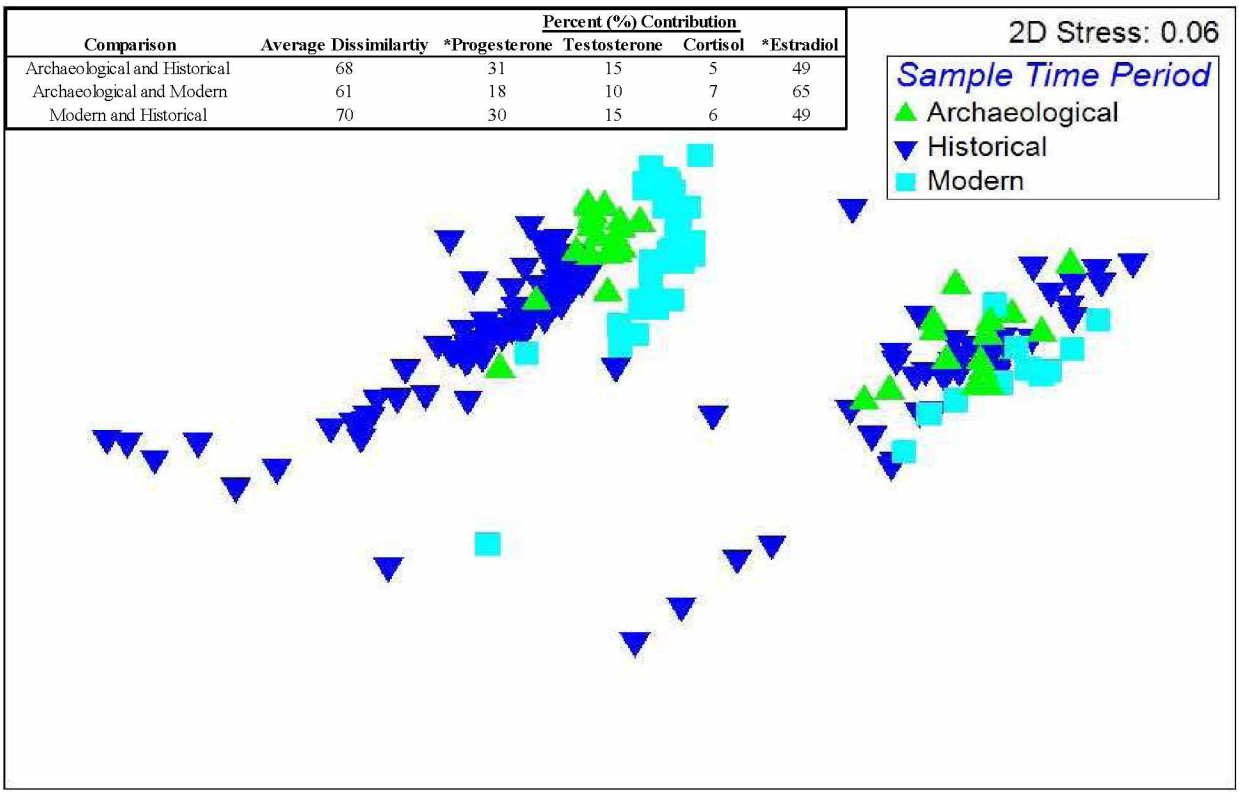


Figure 3.4: **Nonmetric dimensional scaling plot of all walrus samples by sample time period with SIMPER results.** Plot is based on similarities among hormone concentrations among sample time periods (i.e., archaeological, historical, and modern). An inset of the results from a One-Way SIMPER analysis shows which differences among hormone concentration contributed to the average dissimilarity between sample time periods. "*" notes estradiol and progesterone concentration differences among samples contributed the majority to the variability shown in the nMDS plot between sample time periods.

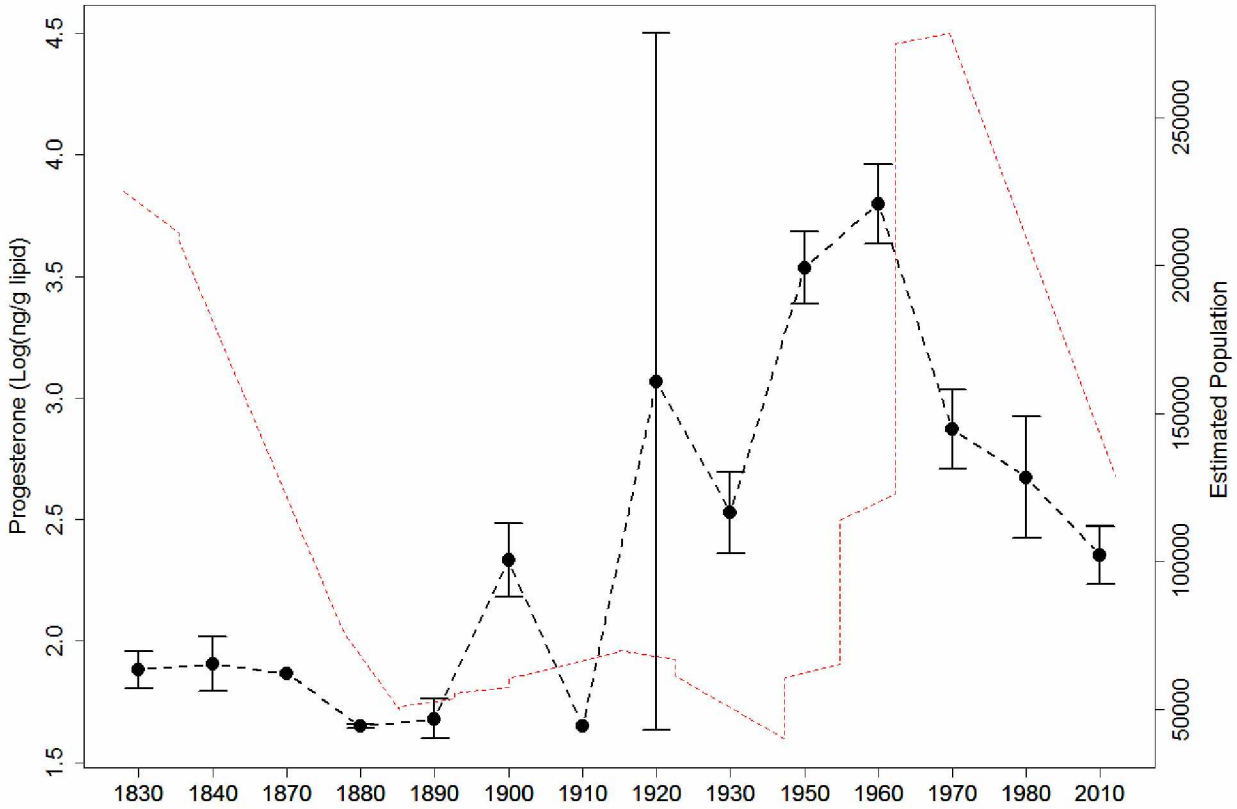


Figure 3.5 A: **The median log transformed progesterone concentrations of each decade for all walrus bones sampled between 1830s – 2010s.** Error bars represent ± 1 SE. Data were unavailable for the 1940s, 1990s, and 2000s. Some decades only have one sample (1870s and 1910s) and only have the one datum point plotted. Estimated population size (Udevitz et al. 2013) is plotted in red to illustrate potential correlations with hormone concentrations. Significant differences in progesterone concentrations among decades are provided in Table 3.8 B.

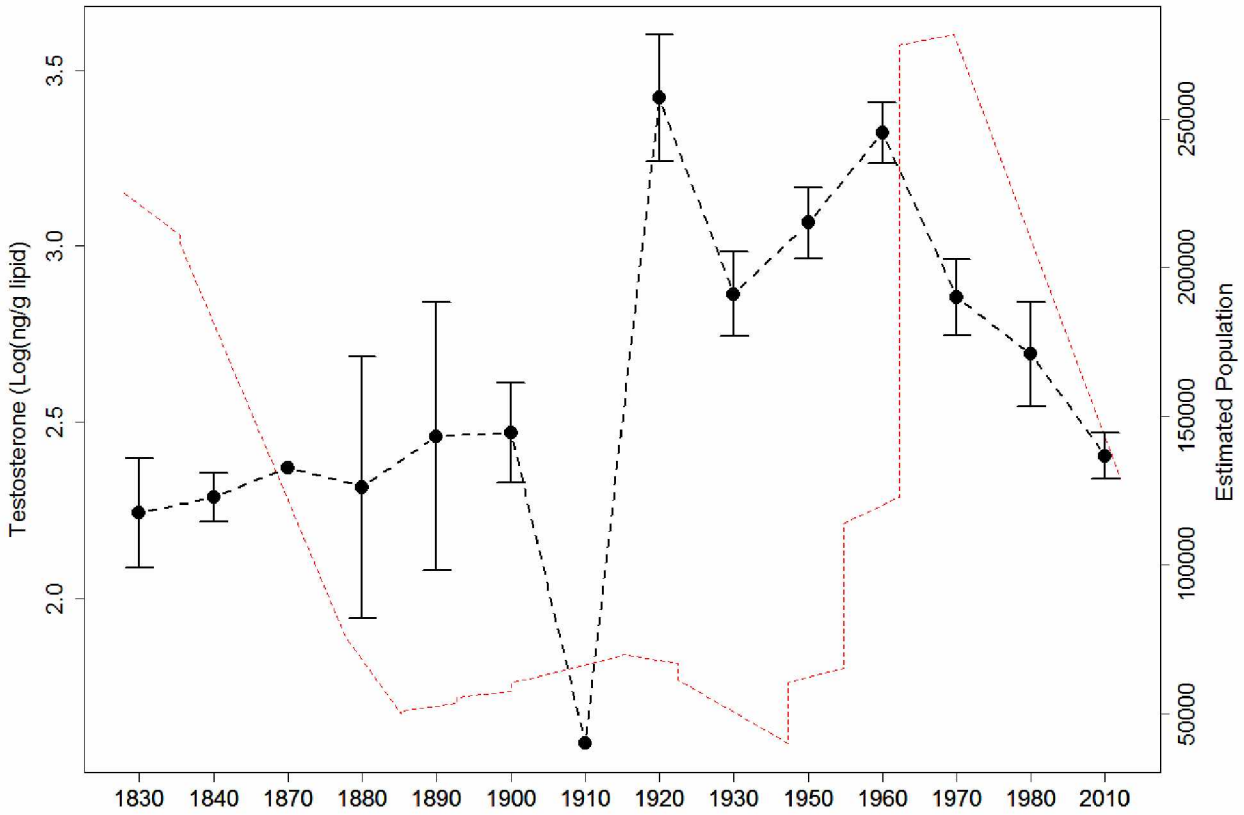


Figure 3.5 B: **The median log transformed testosterone concentrations of each decade for all walrus bones sampled between 1830s – 2010s.** Error bars represent ± 1 SE. Data were unavailable for the 1940s, 1990s, and 2000s. Some decades only have one sample (1870s and 1910s) and only have the one datum point plotted. Estimated population size (Udevitz et al. 2013) is plotted in red to illustrate potential correlations with hormone concentrations. Significant differences in testosterone concentrations among decades are provided in Table 3.8 C.

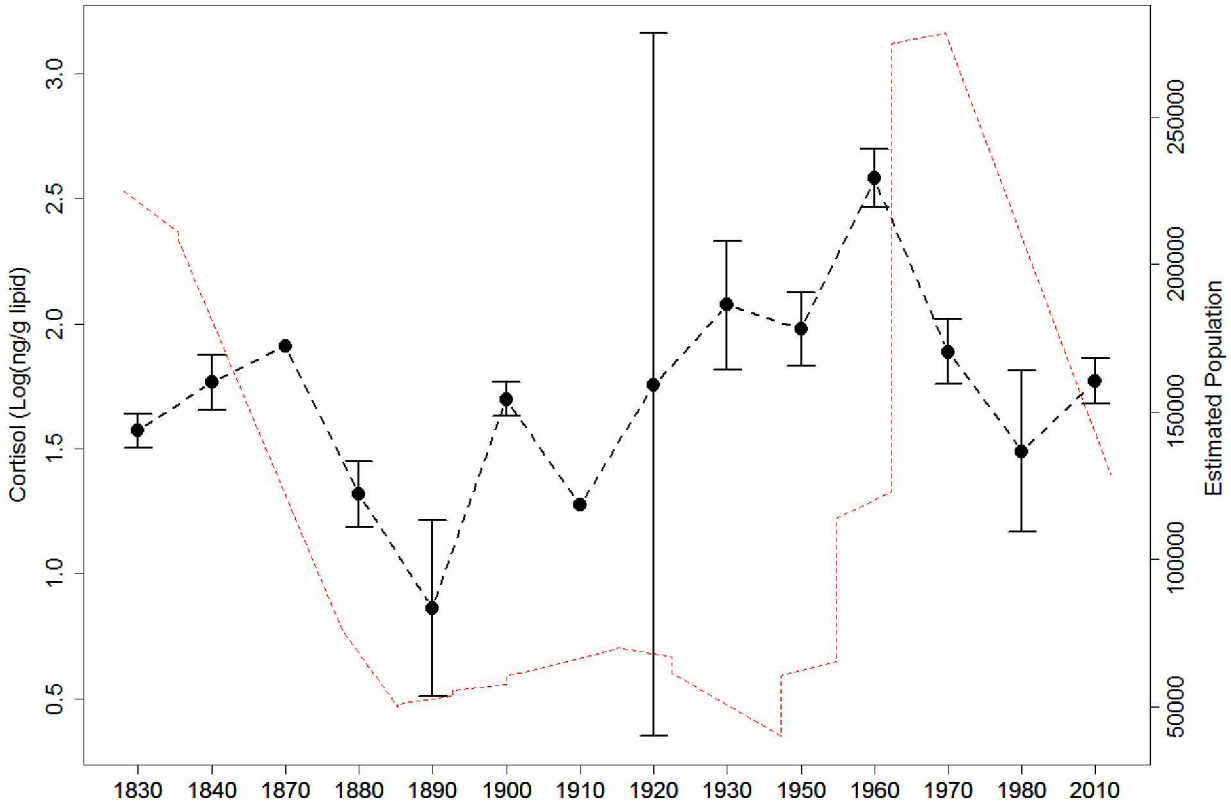


Figure 3.5 C: **The median log transformed cortisol concentrations of each decade for all walrus bones sampled between 1830s – 2010s.** Error bars represent ± 1 SE. Data were unavailable for the 1940s, 1990s, and 2000s. Some decades only have one sample (1870s and 1910s) and only have the one datum point plotted. Estimated population size (Udevitz et al. 2013) is plotted in red to illustrate potential correlations with hormone concentrations. Significant differences in cortisol concentrations among decades are provided in Table 3.8 D.

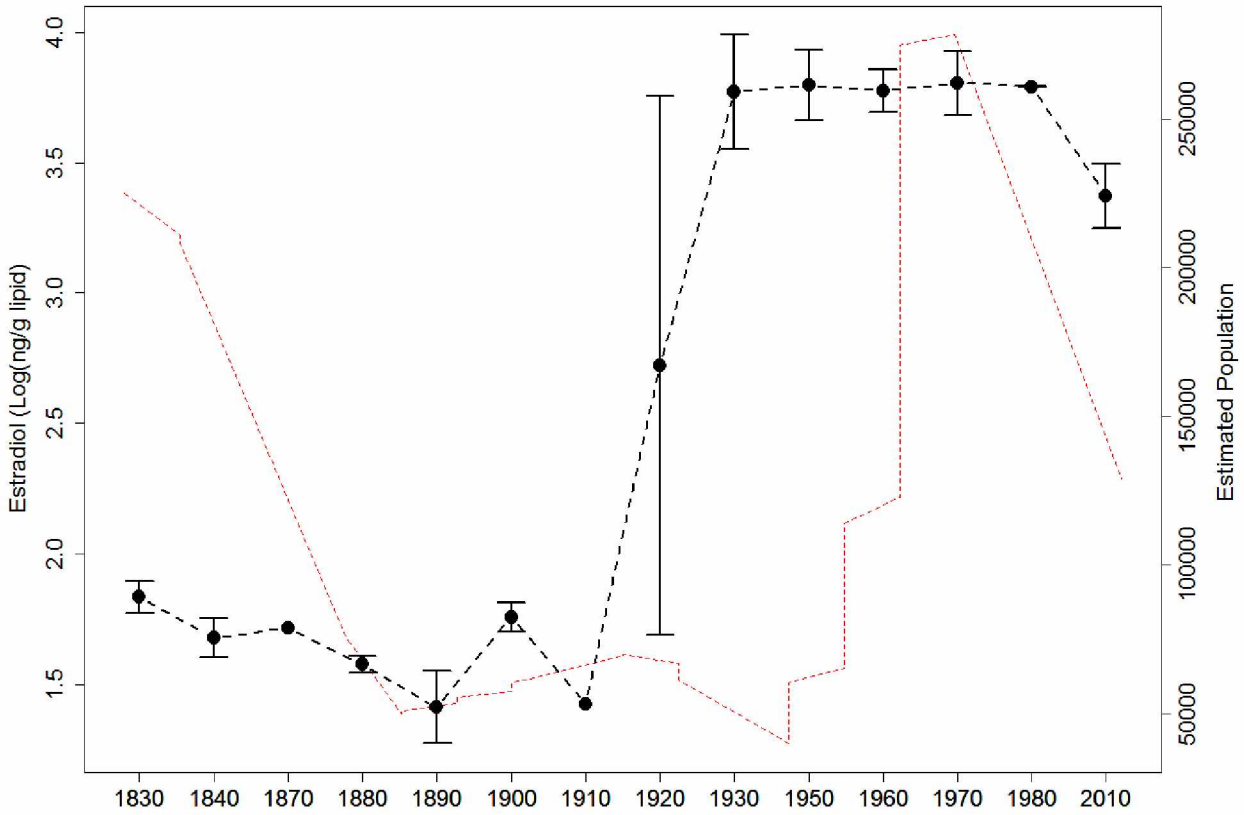


Figure 3.5 D: **The median log transformed estradiol concentrations of each decade for all walrus bones sampled between 1830s – 2010s.** Error bars represent ± 1 SE. Data were unavailable for the 1940s, 1990s, and 2000s. Some decades only have one sample (1870s and 1910s) and only have the one datum point plotted. Estimated population size (Udevitz et al. 2013) is plotted in red to illustrate potential correlations with hormone concentrations. Significant differences in estradiol concentrations among decades are provided in Table 3.8 E.

3.9 Tables

Table 3.1: **Minimum Pacific walrus population estimated from the available literature.** Conservative estimates were used (i.e., minimum populations estimates) due to high variability in historical assessments based on whaling logbooks and models. *The mean estimate from Speckman et al. (2011) was used due to a wide confidence interval in their population estimates. In addition, Speckman et al. (2011) was used as the most recent estimate for 2014 and 2015.

Year	Estimated Minimum Population	Source of Population Estimates
1830	225000	Bockstoce 1995; Hufford and Loughlin 2009 (Figure 6.21)
1850	200000	Bockstoce 1995; Hufford and Loughlin 2009 (Figure 6.21)
1880	50000	Bockstoce 1995; Hufford and Loughlin 2009 (Figure 6.21)
1928	70000	Hufford and Loughlin 2009 (Figure 6.21)
1954	40000	Fay 1957
1960	65500	Fay et al. 1997
1961	75400	Fay et al. 1997
1968	105900	Fay et al. 1997
1972	97700	Fay et al. 1997
1975	220300	Fay et al. 1997
1980	290700	Fay et al. 1997
1985	234020	Hufford and Loughlin 2009 (Fig 6.21)
1990	201039	Gilbert et al. 1992
1997	188316	Hufford and Loughlin 2009 (Fig 6.21)
2006	129000*	Speckman et al. 2011

*The 2006 mean population estimate from Speckman et al. 2011 was used for 2014 and 2015 as a best estimate.

Table 3.2: **Estimated annual Pacific walrus population growth rate for respective years.** These growth rates (Udevitz *et al.* 2013) were applied to the minimum population estimates from the literature (Table 3.1) for respective time periods (years).

Years	Estimated Annual Population Growth Rate
1830 – 1850	0.99
1850 – 1880	0.95
1880 – 1928	1.01
1928 – 1954	0.98
1954 – 1960	1.09
1961 – 1968	1.05
1968 – 1972	1.08
1972 – 1975	1.16
1975 – 1980	1.06
1980 – 1985	0.96
1985 – 1990	0.97
1990 – 1997	0.99
1997 – 2006	0.96
2006 – 2015	1.00

Table 3.3: **Estimated walrus cortical bone turnover rate based on walrus and human skeletal information.** "Calculation (Item Letters)" column shows mathematical calculations used. Dash "-"indicates no calculation were used and information for male and female walruses came from the literature (Source). Dash, "-", in the "Source" column indicates that no source was used, and a mathematical calculation was applied for the specific biological information.

Item	Walrus Biological Information	Calculation (Item Letters)	Male	Female	Source
A	Total Weight [kg]	-	1391.00	774.50	Fay 1982*
B	Skeleton [% of body weight]	-	4.30	3.60	Fay 1982**
C	Skeleton Weight [kg]	A*B	59.81	27.88	-
D	Cortical Bone in Walrus [kg]	0.75*C	44.86	20.91	Clarke 2008 ***
E	Cortical Bone Turnover Rate [%/year]	-	3.00	3.00	Clarke 2008****
F	Cortical Bone Turnover Rate in Walrus [kg/year]	D*0.03 (E)	1.35	0.63	-
G	**Time for Complete Cortical Bone Turnover in Walrus (years)**	D/F	**33.33**	**33.33**	-

*Median weight of adults

**One skeleton weighed for each sex - skeleton is skull with tusks and post cranial skeleton that has been thoroughly cleaned and dried

***Based on human adult skeleton - cortical bone comprises ~75% of the skeleton

****Based on human adult

Table 3.4: **Mean and median steroid hormone concentrations and concentration ranges for all walrus samples by sex and age class.** Steroid hormone concentrations are in ng/g lipid with min and max ranges for each hormone (i.e., cortisol, estradiol, progesterone, and testosterone). Means and medians ± 1 SD are reported."-" indicates no data for available steroid hormone concentrations.

Sex	Age Class	Sample Size (*n*)	Hormone	Mean ± 1 SD (ng/g lipid)	Median (ng/g lipid)	Range: Min – Max (ng/ g lipid)
Female	Adult	36	Progesterone	19501.38 ± 62123.15	1000.42	13.47 – 276407.72
			Testosterone	1834.72 ± 3036.67	892.68	76.68 – 14392.77
			Cortisol	1038.29 ± 2268.38	87.61	14.023 – 10062.76
			Estradiol	5079.82 ± 1935.78	5845.98	29.79 – 7455.46
	Subadult	15	Progesterone	8302.07 ± 7748.50	7006.5	448.79 – 30329.86
			Testosterone	3045.56 ± 2296.40	2629.83	641.17 – 7621.84
			Cortisol	1144.38 ± 2651.43	238.42	40.26 – 10412.57
			Estradiol	7171.29 ± 1245.98	6878.43	5468.22 – 9460.71
	Unknown	1	Progesterone	440.70	-	-
			Testosterone	1162.74	-	-
			Cortisol	59.02	-	-
			Estradiol	2080.20	-	-
Male	Adult	68	Progesterone	3201.82 ± 8587.65	329.13	3.49 – 42873.21
			Testosterone	1305.49 ± 5089.78	313.27	33.19 – 41718.13
			Cortisol	313.91 ± 882.54	56.69	2.24 – 6226.90
			Estradiol	3156.76 ± 2752.03	2368.69	13.48 – 8329.49
	Subadult	25	Progesterone	9764.10 ± 20603.85	3208.73	44.86 – 98533.53
			Testosterone	2365.53 ± 3151.27	1367.22	93.06 – 12644.41
			Cortisol	538.46 ± 977.94	102.24	2.96 – 4077.89
			Estradiol	5743.04 ± 1892.83	6256.99	22.03 – 9754.42
	Unknown	1	Progesterone	225.23	-	-
			Testosterone	255.88	-	-
			Cortisol	169.63	-	-
			Estradiol	2233.05	-	-

Table 3.4 continued:

	Adult	15	Progesterone Testosterone Cortisol Estradiol
Unknown	Subadult	9	Progesterone Testosterone Cortisol Estradiol
	Unknown	50	Progesterone Testosterone Cortisol Estradiol

15373.23 ± 47839.41	514.36	43.75 – 185780.36
1801.56 ± 4171.56	355.33	145.51 – 16379.21
549.39 ± 1268.93	74.72	9.60 – 4910.70
3281.80 ± 2016.07	3696.17	30.35 – 5686.17
1514.88 ± 3053.47	101.24	45.08 – 9230.67
2136.43 ± 2653.61	365.27	44.94 – 7694.14
240.92 ± 592.73	29.61	3.76 – 1816.75
2984.46 ± 2930.28	3948.18	38.04 – 7230.73
173.76 ± 223.16	91.31	20.26 – 1390.73
280.81 ± 293.31	210.08	35.61 – 1803.85
140.61 ± 303.56	52.57	11.86 – 1889.60
1008.21 ± 1862.01	56.71	10.01 – 7161.93

Table 3.5: **Walrus hormone concentration data and results of ANOVAs among time periods.** Mean and median steroid hormone concentrations (i.e., cortisol, estradiol, progesterone, and testosterone) ± 1 SD, concentration ranges, and sample sizes (*n*), for each walrus sample time period (i.e., archaeological, historical, and modern) are reported. Comparison column shows the statistical comparison (PERMANOVAs) of the sample time periods' hormone concentrations with the respective *P* values (bolded *P* values indicate significant differences among sample time periods). Note: significant differences are among the median hormone concentrations and not means.

Sample Time Period	Sample Size (*n*)	Hormone	Mean ± 1 SD Median (ng/g lipid)	Range: Min – Max (ng/ g lipid)	Comparison	*P* value
Archaeological (>200 BP)	38	Progesterone	481.39 ± 1405.28 129.19	20.26 – 8740.18	Modern	0.31
		Testosterone	277.60 ± 328.69 197.66	35.61 – 1803.85		
		Cortisol	157.71 ± 345.32 43.26	11.86 – 1889.60		
		Estradiol	2324.85 ± 2259.20 3503.75	10.01 – 7161.93		
Historical (20 – 200 BP)	135	Progesterone	11075.93 ± 37439.46 743.85	41.70 – 276407.72	Archaeological	0.05
		Testosterone	2085.99 ± 4297.86 895.42	33.19 – 41718.13		
		Cortisol	637.58 ± 1591.60 81.33	2.24 – 10412.57		
		Estradiol	4476.28 ± 3035.53 5844.89	13.69 – 9754.42		
Modern (2014 – 2015)	47	Progesterone	605.70 ± 999.10 225.23	3.49 – 5464.69	**Historical**	**0.04**
		Testosterone	621.23 ± 2076.30 254.52	45.05 – 14392.77		
		Cortisol	318.17 ± 1118.54 59.16	4.64 – 7395.37		
		Estradiol	1847.81 ± 1060.80 2343.49	13.48 – 4030.24		

Table 3.6: **Mean and median walrus bone hormone concentrations with sample size information by decade.** Total walrus sample sizes and sample size by sex with mean and median steroid hormone concentrations (*i.e.*, cortisol, estradiol, progesterone, and testosterone) ± 1 SD (ng/g lipid) among decades 1830 – 2010s. Median values for decades with less than three total samples are not reported.

					Mean ± 1 SD (ng/g lipid) Median			
Decade	**Total Sample Size (*n*)**	**Female (*n*)**	**Male (*n*)**	**Unknown (*n*)**	**Progesterone**	**Testosterone**	**Cortisol**	**Estradiol**
1830	4	0	0	4	84.88 ± 30.43 77.13	158.49 ± 81.44 178.39	35.95 ± 10.29 37.64	66.17 ± 17.51 69.12
1840	8	0	0	8	123.62 ± 108.48 81.04	253.34 ± 145.48 195.22	66.67 ± 51.52 58.62	46.02 ± 16.42 47.84
1870	1	0	0	1	73.65	235.68	81.33	52.19
1880	5	0	1	4	44.77 ± 2.03 44.65	1751.02 ± 3328.63 207.61	23.93 ± 17.40 20.88	36.79 ± 6.10 37,84
1890	3	0	2	1	57.03 ± 20.16 47.87	312.52 ± 291.90 288.83	25.53 ± 33.91 7.29	32.74 ± 18.84 25.92
1900	7	0	0	7	208.04 ± 145.14	637.38 ± 828.87	56.47 ± 22.50	62.69 ± 21.64
1910	1	0	1	0	44.78	38.98	18.85	26.70123
1920	2	0	1	1	15772.94 ± 22245.35	2860.95 ± 1585.51	724.66 ± 1021.65	2867.61 ± 3986.04
1930	13	8	3	2	1233.77 ± 1608.23 337.25	2057.57 ± 2929.38 731.29	645.96 ± 943.90 118.65	4988.41 ± 2215.84 5894.00
1950	28	16	11	0	5655.10 ± 7624.36 3208.73	1716.84 ± 1720.31 1172.02	714.47 ± 1965.14 95.26	5698.48 ± 2375.47 6232.53
1960	34	9	22	3	32309.29 ± 68305.06 6266.90	4074.23 ± 7450.02 2104.18	1109.03 ± 1685.84 385.27	5744.20 ± 2206.76 5950.84
1970	27	8	19	1	6882.14 ± 19803.04 743.85	1668.41 ± 2691.81 716.60	654.80 ± 2099.74 77.21	6039.13 ± 1946.08 6385.93
1980	2	0	2	0	551.48 ± 404.21	522.37 ± 240.61	39.80 ± 35.52	6184.97 ± 39.69
2010	47	11	32	4	606.50 ± 998.62 225.23	621.23 ± 2076.30 254.52	318.17 ± 1118.54 59.16	1847.81 ± 1060.80 2343.49

Table 3.7 A-E: **Differences in steroid hormone concentrations differences by decades 1830s – 2010s via Kruskal-Wallis ANOVAs.** Bolded *P* values indicate significant differences among decades for the respective hormone (A). Mann-Whitney pairwise *post hoc* tests for individual hormones progesterone (B), testosterone (C), cortisol (D), and estradiol (E). Bolded *P* values indicate significant differences among decades.

A)

Kruskal-Wallis ANOVAs	
Hormone (ng/g lipid)	***P* value**
Progesterone	**<0.001**
Testosterone	**<0.001**
Cortisol	**<0.001**
Estradiol	**<0.001**

B)

Progesterone

	1830	1840	1870	1880	1890	1900	1910	1920	1930	1950	1960	1970	1980	2010
1830	-	0.67	0.72	**0.02**	0.22	0.40	0.29	0.82	**0.03**	**0.02**	**0.005**	**0.01**	0.11	0.35
1840	-	-	0.85	0.09	0.36	0.33	0.56	0.90	**0.01**	**0.001**	**<0.001**	**0.002**	0.09	0.32
1870	-	-	-	0.24	1.00	0.66	1.00	0.54	0.21	0.21	0.15	0.17	0.54	0.61
1880	-	-	-	-	0.37	**0.01**	1.00	0.85	**0.003**	**0.002**	**0.001**	**0.002**	0.08	0.19
1890	-	-	-	-	-	0.07	1.00	0.77	**0.02**	**0.02**	**0.01**	**0.01**	0.15	0.35
1900	-	-	-	-	-	-	0.19	0.88	0.13	**0.003**	**<0.001**	**0.04**	0.31	0.76
1910	-	-	-	-	-	-	-	0.54	0.14	0.14	0.12	0.17	0.54	0.56
1920	-	-	-	-	-	-	-	-	0.93	0.97	0.76	0.97	0.70	0.46
1930	-	-	-	-	-	-	-	-	-	**0.01**	**0.001**	0.77	0.67	0.06
1950	-	-	-	-	-	-	-	-	-	-	0.19	**0.03**	0.15	**<0.001**
1960	-	-	-	-	-	-	-	-	-	-	-	**0.002**	0.12	**<0.001**
1970	-	-	-	-	-	-	-	-	-	-	-	-	0.97	**0.006**
1980	-	-	-	-	-	-	-	-	-	-	-	-	-	0.43
2010	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3.7 A-E continued:

C) **Testosterone**

	1830	1840	1870	1880	1890	1900	1910	1920	1930	1950	1960	1970	1980	2010
1830	-	0.44	0.29	0.71	0.60	0.03	0.29	0.11	**0.004**	**0.01**	**0.003**	**0.02**	0.11	0.21
1840	-	-	0.85	0.61	0.76	0.07	0.18	0.05	**<0.001**	**0.002**	**<0.001**	**0.02**	0.09	0.95
1870	-	-	-	1.00	1.00	0.38	1.00	0.54	0.14	0.26	0.15	0.32	0.54	0.89
1880	-	-	-	-	1.00	0.75	0.24	0.33	0.14	0.15	0.05	0.30	0.56	0.78
1890	-	-	-	-	-	0.82	1.00	0.15	0.06	**0.03**	**0.01**	0.13	0.39	0.93
1900	-	-	-	-	-	-	0.19	0.11	**0.01**	**0.03**	**0.003**	0.14	0.46	0.16
1910	-	-	-	-	-	-	-	0.54	0.14	0.11	0.10	0.11	0.54	0.10
1920	-	-	-	-	-	-	-	-	0.20	0.17	0.56	0.11	0.25	**0.02**
1930	-	-	-	-	-	-	-	-	-	0.42	0.05	0.40	0.27	**<0.001**
1950	-	-	-	-	-	-	-	-	-	-	0.05	0.17	0.17	**<0.001**
1960	-	-	-	-	-	-	-	-	-	-	-	**0.003**	**0.06**	**<0.001**
1970	-	-	-	-	-	-	-	-	-	-	-	-	0.58	**<0.001**
1980	-	-	-	-	-	-	-	-	-	-	-	-	-	0.16
2010	-	-	-	-	-	-	-	-	-	-	-	-	-	-

D) **Cortisol**

	1830	1840	1870	1880	1890	1900	1910	1920	1930	1950	1960	1970	1980	2010
1830	-	0.27	0.29	0.27	0.60	0.11	0.29	0.82	0.23	**0.04**	**0.005**	0.11	0.82	0.18
1840	-	-	0.33	0.05	0.18	0.95	0.33	0.90	0.37	0.09	**0.001**	0.38	0.51	0.78
1870	-	-	-	0.24	0.37	0.38	1.00	0.54	1.00	0.77	0.25	0.80	0.54	0.61
1880	-	-	-	-	0.55	**0.03**	1.00	0.85	0.05	**0.01**	**0.001**	**0.01**	0.56	**0.02**
1890	-	-	-	-	-	0.25	1.00	0.77	0.14	**0.05**	**0.01**	0.06	0.39	0.09
1900	-	-	-	-	-	-	0.19	0.88	0.34	0.07	**0.001**	0.44	0.66	0.70
1910	-	-	-	-	-	-	-	0.54	0.46	0.17	0.12	0.17	0.54	0.28
1920	-	-	-	-	-	-	-	-	0.67	0.84	0.65	0.90	0.70	0.94
1930	-	-	-	-	-	-	-	-	-	0.99	0.14	0.58	0.44	0.30
1950	-	-	-	-	-	-	-	-	-	-	**0.01**	0.28	0.15	**0.06**
1960	-	-	-	-	-	-	-	-	-	-	-	**0.0004**	0.06	**<0.001**
1970	-	-	-	-	-	-	-	-	-	-	-	-	0.25	0.51
1980	-	-	-	-	-	-	-	-	-	-	-	-	-	0.40
2010	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3.7 A-E continued:

E) **Estradiol**

	1830	**1840**	**1870**	**1880**	**1890**	**1900**
1830	-	0.15	0.72	**0.04**	0.11	0.64
1840	-	-	0.56	0.12	0.61	0.18
1870	-	-	-	0.24	1.00	1.00
1880	-	-	-	-	0.55	**0.02**
1890	-	-	-	-	-	0.11
1900	-	-	-	-	-	-
1910	-	-	-	-	-	-
1920	-	-	-	-	-	-
1930	-	-	-	-	-	-
1950	-	-	-	-	-	-
1960	-	-	-	-	-	-
1970	-	-	-	-	-	-
1980	-	-	-	-	-	-
2010	-	-	-	-	-	-

1910	1920	1930	1950	1960	1970	1980	2010
0.29	0.82	**0.03**	**0.01**	**0.003**	**0.007**	0.11	**0.06**
0.33	0.36	**0.002**	**<0.001**	**<0.001**	**<0.001**	0.05	**0.01**
1.00	0.54	0.32	0.17	0.12	0.17	0.54	0.35
0.24	0.08	**0.002**	**0.001**	**0.001**	**0.003**	0.08	**0.03**
1.00	0.39	**0.02**	**0.01**	**0.01**	**0.01**	0.15	**0.03**
0.19	0.88	**0.01**	**0.001**	**<0.001**	**0.001**	0.06	**0.02**
-	0.54	0.14	0.14	0.10	0.14	0.54	0.19
-	-	0.35	0.12	0.18	0.08	0.25	0.63
-	-	-	**0.03**	0.22	**0.01**	0.15	**<0.001**
-	-	-	-	0.51	0.74	0.71	**<0.001**
-	-	-	-	-	0.31	0.86	**<0.001**
-	-	-	-	-	-	0.46	**<0.001**
-	-	-	-	-	-	-	**0.02**
-	-	-	-	-	-	-	-

Table 3.8: **Percent contribution to the dissimilarities in steroid hormone concentrations among decades significantly different from the 2010s.** Includes only decades with steroid hormone concentrations (i.e., cortisol, estradiol, progesterone, and testosterone) sample size $n >$ 10. Mean percent dissimilarity is based on a scale of 0 % = similar and 100 % = no similarities.

		Percent (%) Contribution			
Decade Comparison	**Average Dissimilartiy**	**Progesterone**	**Testosterone**	**Cortisol**	**Estradiol**
1930: 2010	64	14	23	8	55
1950: 2010	70	36	13	6	45
1960: 2010	74	48	13	6	33
1970: 2010	65	24	11	4	61
1980: 2010	57	10	8	3	79

Table 3.9: **Spearman's rank correlation of steroid hormones compared with estimated Pacific walrus population size.** Significant *P* values and *r* values are bolded. Males were pooled with female walruses, but females were also run separately, because progesterone and estradiol are important female reproductive hormones, and sex sampled in certain decades could therefore skew the analysis.

Hormone	*r* value	*P* value	*r* value (females only)	*P* value (females only)
Progesterone	**-0.21**	**0.003**	**-0.42**	**0.002**
Testosterone	**-0.29**	**<0.001**	-0.25	0.07
Cortisol	**-0.15**	**0.04**	-0.20	0.13
Estradiol	-0.07	0.32	**-0.36**	**0.008**

Appendix 3.1: **List of archaeological walrus specimens with their respective provenience and steroid hormone concentrations data.** Archaeological time period is defined as bones with ages greater than 200 years before present (BP). Samples were collected from various archaeological collections from the University of Alaska (UAM: ARCH), Sanak Island excavation (Sanak), and collections curated by Dr. A. Jensen at Ukpeaġvik Iñupiat Corporation (Barrow). Carbon dates of bones are still being determined for a subset of samples (TBD). Samples with ranges of calibrated dates were determined by using the earliest and latest radiocarbon dates respective to the archaeological excavation where the walrus bone was found (i.e., radiocarbon dates of the general site, not specifically the walrus bone). Hormone concentrations (ng/g lipid) abbreviated as follows: progesterone (P), testosterone (T), cortisol (C), and estradiol (E). Sexes were unknown for all individuals.

Archaeological Samples													
Project Number	Catalog Number	Source	Skeletal Element	Site	Unit	Depth	Area	Age Class	P [ng/g lipid]	T [ng/ g lipid]	C [ng/g lipid]	E [ng/g lipid]	Median Calibrate Before Present [BP]
WAL158	VL112	Sanak	Mandible	XFP-063	TP 3	< 30 cm	Sanak Island	Unknown	305.23	113.24	29.61	4621.10	3585
WAL163	VL129	Sanak	Fragment	XFP-095	Unknown	Unknown	Sanak Island	Unknown	34.04	149.88	11.86	4369.93	927
WAL165	SL2-3420	Barrow	Tibia	SL-2	Unknown	Unknown	Pingasasruk	Subadult	101.24	212.59	27.84	3948.18	565 – 215
WAL166	SL2-3163(0)	Barrow	Vertebrae	SL-2	Unknown	Unknown	Pingasaruk	Unknown	101.07	128.99	744.69	4391.03	565 – 215
WAL167	SL2-3429(0)	Barrow	Rib	SL-2	Unknown	Unknown	Pingasasruk	Unknown	197.00	60.62	30.31	3840.30	565 – 215
WAL168	SL2-3495(0)	Barrow	Rib	SL-2	Unknown	Unknown	Pingasasruk	Unknown	139.23	335.70	484.04	4166.37	565 – 215
WAL169	SL2-3522(0)	Barrow	Skull	SL-2	Unknown	Unknown	Pingasasruk	Unknown	85.43	206.05	1889.60	4089.53	565 – 215
WAL170	SL2-CQDQL	Barrow	Radius	SL-2	Unknown	Unknown	Pingasaruk	Unknown	57.62	103.18	862.97	4141.97	565 – 215
WAL171	SL2-KAIMH	Barrow	Mandible	SL-2	Unknown	Unknown	Pingasasruk	Unknown	561.96	1317.14	348.54	3839.04	400 ± 24
WAL172	UA72-060-0040	UAM: ARCH	Mandible	Okvik Hillside	Unknown	Unknown	Gambell	Unknown	371.69	337.34	76.06	4168.33	976 – 634
WAL174	UA72-060-0042	UAM: ARCH	Mandible	Okvik Hillside	Unknown	Unknown	Gambell	Unknown	1390.73	1803.85	167.30	7161.93	976 – 634
WAL175	UA72-060-0045	UAM: ARCH	Mandible	Okvik Hillside	Unknown	Unknown	Gambell	Unknown	632.63	266.10	181.31	4308.43	976 – 634
WAL176	UA72-065-0525	UAM: ARCH	Mandible	Okvik Hillside	Unknown	Unknown	Kitnigipaluk	Adult	514.36	238.54	41.00	4214.26	1515 – 915
WAL177	UA72-065-0526	UAM: ARCH	Mandible	Okvik Hillside	Unknown	Unknown	Kitnigipaluk	Adult	8740.18	501.54	112.01	4196.48	1515 – 915
WAL178	UA72-065-0527	UAM: ARCH	Mandible	Okvik Hillside	Unknown	Unknown	Kitnigipaluk	Adult	706.12	274.52	41.10	5006.00	1515 – 915
WAL179	UA72-065-0528	UAM: ARCH	Mandible	Okvik Hillside	Unknown	Unknown	Kitnigipaluk	Subadult	248.37	139.78	19.64	3770.56	1515 – 915
WAL180	UA72-065-0529	UAM: ARCH	Mandible	Okvik Hillside	Unknown	Unknown	Kitnigipaluk	Adult	837.68	199.76	75.05	3696.17	1515 – 915
WAL181	UA72-065-0530	UAM: ARCH	Mandible	Okvik Hillside	Unknown	Unknown	Kitnigipaluk	Adult	231.32	153.55	24.93	3311.33	1515 – 915
WAL182	UA72-065-0531	UAM: ARCH	Mandible	Okvik Hillside	Unknown	Unknown	Kitnigipaluk	Adult	514.99	145.51	35.66	4499.37	1515 – 915
WAL183	UA72-065-0534	UAM: ARCH	Mandible	Okvik Hillside	Unknown	Unknown	Kitnigipaluk	Adult	567.24	211.26	38.26	5599.22	1515 – 915
WAL242	UA75-009 -- XRH-00001	UAM:ARCH	Humerus	Old Tigara	S10 W8	100-160	Point Hope	Unknown	20.26	233.10	71.61	44.04	TBD
WAL245	UA75-009 -- XRH-00001	UAM:ARCH	Humerus Fragment	Old Tigara	s14w8	0-50	Point Hope	Unknown	27.36	217.56	62.66	19.49	TBD
WAL249	UA75-009 -- XPH-001	UAM:ARCH	Cranial Fragment	Old Tigara	n0e4	90-100	Point Hope	Unknown	31.11	102.53	57.57	27.52	TBD
WAL255	UA75-009 -- XPH-001	UAM:ARCH	Rib	Old Tigara	s8w12	120-130	Point Hope	Unknown	53.85	172.31	59.46	38.16	390 ± 23
WAL257	UA75-009 -- XRH-00001	UAM:ARCH	Cranial Fragment	Old Tigara	s12w8	86-96	Point Hope	Unknown	58.60	211.17	47.27	39.50	TBD
WAL259	UA75-009 -- XPH-001	UAM:ARCH	Cranial Fragment	Old Tigara	n4e4	120-130	Point Hope	Unknown	271.28	185.10	35.01	56.41	TBD
WAL262	UA75-009 -- XPH-001	UAM:ARCH	Skull Fragment	Old Tigara	s8w18	110-150?	Point Hope	Unknown	108.25	536.85	60.70	199.80	TBD
WAL263	UA75-009 -- XRH-00001	UAM:ARCH	Bulae	Old Tigara	s8w12	135-165	Point Hope	Unknown	119.15	152.77	51.87	10.01	300 ± 22
WAL265	UA75-009 -- XPH-001	UAM:ARCH	Cranial Fragment	Old Tigara	N0e4	110-120	Point Hope	Unknown	44.14	227.76	30.15	38.94	TBD
WAL266	UA75-009 -- XPH-001	UAM:ARCH	Rib	Old Tigara	s14w8	0-50	Point Hope	Unknown	73.56	163.12	40.48	59.23	TBD
WAL267	UA75-009 -- XRH-00001	UAM:ARCH	Femur	Old Tigara	n4e4	150-160	Point Hope	Unknown	46.26	241.16	54.67	125.88	TBD
WAL268	UA75-009 -- XPH-001	UAM:ARCH	Femur	Old Tigara	s8w12	135-165	Point Hope	Unknown	89.32	117.12	29.52	35.07	300 ± 22
WAL269	UA75-009 -- XRH-00001	UAM:ARCH	Cranial Fragment	Old Tigara	s10w8	70-100	Point Hope	Unknown	103.56	170.11	24.95	33.88	TBD
WAL270	UA75-009 -- XPH-001	UAM:ARCH	Rib	Old Tigara	s6w18	94-105	Point Hope	Unknown	47.18	35.61	24.01	57.01	TBD
WAL272	UA75-009 -- XPH-001	UAM:ARCH	Rib	Old Tigara	s6w12	80-100	Point Hope	Unknown	253.20	195.55	45.41	48.99	TBD
WAL275	UA75-009 -- XPH-001	UAM:ARCH	Maxilla Fragment	Old Tigara	n6e4	110-130	Point Hope	Unknown	244.98	400.85	21.35	74.29	TBD
WAL277	UA75-009 -- XPH-001	UAM:ARCH	Max Fragment	Old Tigara	n0e0	70-80	Point Hope	Unknown	92.08	154.41	16.71	60.19	TBD
WAL280	UA75-009 -- XPH-001	UAM:ARCH	Cranial Fragment	Old Tigara	s12w8	100-135	Point Hope	Unknown	270.69	132.70	17.68	36.22	TBD

Appendix 3.2: **List of historical walrus bone samples and their respective provenience and steroid hormone concentrations data.** Historical time period defined as bones aged 200 years before present (BP) to 20 BP. Samples were collected from University of Alaska Museum (UAM: Mamm), the Smithsonian Institute (Smith: Mamm), and University of Alaska Archaeological Collections (UAM: ARCH). *Indicates collected dates determined by Atomic Mass Spectrometry (AMS) radiocarbon dating techniques described in methods (± Error). **Indicates specimen had duplicate samples analyzed for steroid hormones (progesterone (P), testosterone (T), cortisol (C), and estradiol (E)), and duplicate concentrations were averaged to determine final concentrations shown (ng/g lipid).

Historical Samples

Project Number	Catalog Number	Source	Location	Sex	Age Class	Year Collected	P [ng/g lipid]	T [ng/ g lipid]	C [ng/g lipid]	E [ng/g lipid]
WAL253	UA75-009--XPH-00001	UAM: ARCH	Point Hope	Unknown	Unknown	1830*	59.30	145.59	45.66	44.13
WAL271	UA75-009--XPH-00001	UAM: ARCH	Point Hope	Unknown	Unknown	1830*	64.53	48.70	32.84	77.98
WAL274	UA75-009--XPH-00001	UAM: ARCH	Point Hope	Unknown	Unknown	1830*	89.73	228.49	42.44	60.26
WAL278	UA75-009--XPH-00001	UAM: ARCH	Point Hope	Unknown	Unknown	1830*	125.95	211.19	22.85	82.32
WAL251	UA75-009--XPH-00001	UAM: ARCH	Point Hope	Unknown	Unknown	1841 ± 22*	43.59	176.02	53.27	51.65
WAL247	UA75-009--XPH-00001	UAM: ARCH	Point Hope	Unknown	Unknown	1842 ± 21*	70.57	246.33	63.97	46.15
WAL250	UA75-009--XPH-00001	UAM: ARCH	Point Hope	Unknown	Unknown	1842 ± 21*	42.55	140.24	78.75	37.64
WAL264	UA75-009--XPH-00001	UAM: ARCH	Point Hope	Unknown	Unknown	1842 ± 21*	162.96	208.95	70.95	13.69
WAL254	UA75-009--XPH-00001	UAM: ARCH	Point Hope	Unknown	Unknown	1843 ± 22*	91.51	589.35	182.69	52.60
WAL261	UA75-009--XPH-00001	UAM: ARCH	Point Hope	Unknown	Unknown	1843 ± 22*	138.41	309.41	40.82	72.16
WAL279	UA75-009--XPH-00001	UAM: ARCH	Point Hope	Unknown	Unknown	1843 ± 22*	69.11	174.89	18.74	44.77
WAL281	UA75-009--XPH-00001	UAM: ARCH	Point Hope	Unknown	Unknown	1843 ± 22*	370.24	181.50	24.18	49.53
WAL256	UA75-009--XPH-00001	UAM: ARCH	Point Hope	Unknown	Unknown	1871 ± 21 *	73.65	235.68	81.33	52.19
WAL228.2	USNM16447	Smith: Mamm	Big Diomede Island	Unknown	Subadult	1880	47.51	7694.14	52.94	38.04
WAL229.1	USNM16437	Smith: Mamm	Plover Bay	Male	Adult	1880	42.16	207.61	20.88	31.93
WAL231.2	USNM16448	Smith: Mamm	Big Diomede Island	Unknown	Adult	1880	43.75	601.31	11.51	30.35
WAL217.2	USNM16756	Smith: Mamm	Point Barrow	Unknown	Adult	1883	44.65	207.09	9.60	37.84
WAL218.2	USNM16757	Smith: Mamm	Point Barrow	Unknown	Subadult	1883	45.79	44.94	24.73	45.79
WAL215.2	USNM38981	Smith: Mamm	Pribolof Island	Male	Adult	1890	43.08	33.19	4.65	18.25
WAL258	UA75-009--XPH-00001	UAM: ARCH	Point Hope	Unknown	Unknown	1890 ± 22*	80.14	288.83	64.65	54.03
WAL216	USNM63302	Smith: Mamm	St. Paul Island	Male	Adult	1895	47.87	615.54	7.29	25.92
WAL232.2	USNM108344	Smith: Mamm	East Cape, Siberia	Unknown	Adult	1900	49.20	2497.51	36.46	49.20
WAL244	UA75-009--XPH-00001	UAM: ARCH	Point Hope	Unknown	Unknown	1900 ± 23*	48.02	411.49	93.13	50.33
WAL252	UA75-009--XPH-00001	UAM: ARCH	Point Hope	Unknown	Unknown	1900 ± 23*	91.11	188.22	49.95	57.32
WAL273	UA75-009--XPH-00001	UAM: ARCH	Point Hope	Unknown	Unknown	1900 ± 23*	346.31	267.47	62.11	67.00
WAL248	UA75-009--XPH-00001	UAM: ARCH	Point Hope	Unknown	Unknown	1903 ± 21*	215.50	295.51	76.52	36.19
WAL276	UA75-009--XPH-00001	UAM: ARCH	Point Hope	Unknown	Unknown	1903 ± 21*	335.07	548.27	29.20	101.61
WAL260	UA75-009--XPH-00001	UAM: ARCH	Point Hope	Unknown	Unknown	1907 ± 22*	371.05	253.17	47.88	77.15
WAL219.2	USNM220151	Smith: Mamm	Round Island	Male	Adult	1917	44.78	38.98	18.85	26.70
WAL222.2	USNM248161	Smith: Mamm	St. Paul Island	Male	Adult	1927	43.11	1739.82	2.24	49.06
WAL113	5010	UAM: Mamm	St. Lawrence	Unknown	Unknown	1928	31502.78	3982.08	1447.07	5686.17
WAL223	USNM256001	Smith: Mamm	St. Lawrence Island	Unknown	Subadult	1930	82.65	365.27	3.76	46.32

Apprendix 3.2 continued:

WAL224.2	USNM256002	Smith: Mamm	St. Lawrence Island
WAL050.2	3382	UAM: Mamm	Savoonga
WAL042.2	16592	UAM: Mamm	St. Lawrence
WAL045.2	16593	UAM: Mamm	St. Lawrence
WAL064	16591	UAM: Mamm	St. Lawrence
WAL023.2	16588	UAM: Mamm	St. Lawrence
WAL069	16586	UAM: Mamm	St. Lawrence/Nushagak Bay
WAL121**	16590	UAM: Mamm	St. Lawrence
WAL122.2	16589	UAM: Mamm	St. Lawrence
WAL123.2	16587	UAM: Mamm	St. Lawrence
WAL061	2085	UAM: Mamm	St. Lawrence
WAL118	5220	UAM: Mamm	St. Lawrence
WAL036	5012	UAM: Mamm	Kotzebue
WAL027	11690	UAM: Mamm	St. Lawrence
WAL028	11473	UAM: Mamm	St. Lawrence
WAL029	11514	UAM: Mamm	St. Lawrence
WAL030.1**	11513	UAM: Mamm	St. Lawrence
WAL031.2	11700	UAM: Mamm	St. Lawrence
WAL048	11702	UAM: Mamm	St. Lawrence
WAL053	11515	UAM: Mamm	St. Lawrence
WAL057.2	11696	UAM: Mamm	St. Lawrence
WAL060	11703	UAM: Mamm	St. Lawrence
WAL085.2	11694	UAM: Mamm	St. Lawrence
WAL024	11516	UAM: Mamm	St. Lawrence/Teller
WAL041	3377	UAM: Mamm	St. Lawrence
WAL054	11682	UAM: Mamm	St. Lawrence
WAL065	11691	UAM: Mamm	St. Lawrence
WAL068	11688	UAM: Mamm	St. Lawrence
WAL094	11695	UAM: Mamm	Savoonga
WAL035	11518	UAM: Mamm	St. Lawrence
WAL062	11704	UAM: Mamm	St. Lawrence
WAL081.1	11689	UAM: Mamm	St. Lawrence
WAL086	11710	UAM: Mamm	St. Lawrence
WAL091	11705	UAM: Mamm	St. Lawrence
WAL096	11706	UAM: Mamm	Savoonga
WAL119	11707	UAM: Mamm	St. Lawrence
WAL220.2	USNM287992	Smith: Mamm	Bristol Bay
WAL221.2	USNM287993	Smith: Mamm	Bristol Bay
WAL225.2	USNM287994	Smith: Mamm	Bristol Bay
WAL056	11698	UAM: Mamm	St. Lawrence

Unknown	Subadult	1930	45.08	4321.15	11.73	45.08
Male	Subadult	1931-1932	4221.73	10815.77	818.29	5498.70
Female	Adult	1932	1311.78	589.53	16.70	6144.99
Female	Adult	1932	556.99	585.93	1765.01	5736.28
Female	Adult	1932	316.43	731.29	62.58	5987.54
Female	Adult	1933	281.04	586.16	57.13	5964.97
Female	Adult	1933	329.29	668.37	28.98	6122.91
Female	Adult	1933	843.04	1103.42	422.43	5894.00
Female	Adult	1933	2596.20	889.94	2975.93	5597.23
Female	Adult	1933	4796.57	3916.68	1777.30	6318.86
Male	Adult	1934	321.00	1584.70	118.65	6271.87
Male	Adult	1934	337.25	590.18	338.93	5220.59
Female	Adult	1954	923.75	1330.11	10062.76	7146.99
Male	Unknown	1956	7232.00	1390.34	486.45	6225.74
Female	Unknown	1956	8686.11	3002.19	164.64	6878.43
Female	Subadult	1956	8665.99	2445.95	2638.75	9256.06
Female	Subadult	1956	7717.21	2741.71	1050.27	6271.99
Female	Adult	1956	1257.43	986.16	67.61	6918.77
Female	Adult	1956	3673.68	1136.22	69.98	6635.50
Female	Subadult	1956	14409.69	6367.67	317.79	5786.77
Female	Unknown	1956	448.79	641.17	66.62	6567.48
Male	Adult	1956	700.09	1172.02	95.26	6367.08
Male	Unknown	1956	82.68	165.48	27.54	6232.53
Male	Subadult	1957	2289.16	1932.89	1832.51	6444.28
Male	Adult	1957	10095.83	2524.20	401.31	7555.59
Female	Subadult	1957	17892.16	7396.98	353.39	8089.23
Female	Unknown	1957	3063.16	1268.59	95.30	6207.78
Male	Unknown	1957	36203.42	2328.52	180.12	5685.39
Male	Unknown	1957	3208.73	623.49	34.30	1965.42
Female	Unknown	1958	7006.50	2629.83	88.40	7874.43
Female	Unknown	1958	1837.56	1160.74	120.98	5386.80
Female	Unknown	1958	1605.78	665.94	40.26	6091.02
Female	Adult	1958	1077.09	756.63	89.02	5518.98
Female	Adult	1958	6518.97	1049.07	52.63	5982.16
Male	Unknown	1958	4262.04	1435.97	61.83	6667.29
Female	Adult	1958	3695.63	995.76	865.96	5983.84
Male	Adult	1958	42.40	43.63	3.44	42.40
Male	Adult	1958	46.96	70.42	20.71	54.85
Male	Subadult	1958	44.86	93.06	2.96	22.03
Female	Subadult	1959	6279.25	1077.95	100.99	7692.44

Appendix 3.2 continued:

WAL117	5218	UAM: Mamm	Ogotoruk Creek, Chukchi Sea
WAL018.2	11519	UAM: Mamm	St. Lawrence
WAL022	11701	UAM: Mamm	St. Lawrence
WAL051	4861	UAM: Mamm	Barrow
WAL097	5042	UAM: Mamm	Point Hope
WAL098	5039	UAM: Mamm	Point Hope
WAL099	5044	UAM: Mamm	Point Hope
WAL100	5038	UAM: Mamm	Point Hope
WAL101	5040	UAM: Mamm	Point Hope
WAL102	5041	UAM: Mamm	Point Hope
WAL103	5048	UAM: Mamm	Point Hope
WAL104	5051	UAM: Mamm	Point Hope
WAL105	5043	UAM: Mamm	Point Hope
WAL106	5052	UAM: Mamm	Point Hope
WAL107	5045	UAM: Mamm	Point Hope
WAL108	5047	UAM: Mamm	Point Hope
WAL109	5049	UAM: Mamm	Point Hope
WAL110	5050	UAM: Mamm	Point Hope
WAL112	5061	UAM: Mamm	Point Hope
WAL114	4816	UAM: Mamm	Elephant Point
WAL115	5221	UAM: Mamm	Point Hope
WAL116	5222	UAM: Mamm	Point Hope
WAL043	11517	UAM: Mamm	Teller
WAL044.2**	10522	UAM: Mamm	Teller
WAL075	11686	UAM: Mamm	St. Lawrence
WAL226.2	USNM324983	Smith: Mamm	St. Lawrence Island
WAL021	11711	UAM: Mamm	St. Matthew
WAL039.1**	7277	UAM: Mamm	St. Lawrence
WAL026	11521	UAM: Mamm	St. Lawrence
WAL034**	11522	UAM: Mamm	St. Lawrence
WAL047.2	11512	UAM: Mamm	St. Lawrence
WAL077.1**	11683	UAM: Mamm	St. Lawrence
WAL063	11693	UAM: Mamm	St. Lawrence
WAL072.1	11699	UAM: Mamm	St. Lawrence
WAL025**	11709	UAM: Mamm	St. Lawrence
WAL120	11708	UAM: Mamm	St. Lawrence
WAL111	11637	UAM: Mamm	Bering Sea
WAL055.1	11684	UAM: Mamm	Bering Sea
WAL059	11685	UAM: Mamm	Bering Sea
WAL033	10538	UAM: Mamm	Kotzebue

Unknown	Unknown	1960	485.12	851.46	634.00	5180.70
Female	Subadult	1961	3359.16	2649.88	1010.87	6922.15
Male	Subadult	1961	30677.01	6901.02	2660.16	5208.93
Male	Subadult	1961	17042.73	1964.39	287.24	9754.42
Female	Adult	1961	234235.94	5024.19	259.69	5797.95
Male	Adult	1961	1517.00	741.84	159.92	7114.12
Female	Adult	1961	8236.23	1726.85	162.83	6465.15
Male	Adult	1961	715.53	567.19	109.95	5609.04
Male	Adult	1961	490.11	1060.29	1014.19	8329.49
Male	Adult	1961	2398.83	1687.80	6226.90	5840.86
Female	Adult	1961	131114.23	4796.18	6411.66	6199.49
Female	Adult	1961	276407.72	11853.62	714.99	6955.87
Male	Adult	1961	57.78	99.37	57.98	657.42
Male	Adult	1961	10250.91	1496.18	875.64	5383.15
Male	Adult	1961	32322.83	1168.36	246.46	615.65
Male	Adult	1961	584.03	1330.20	335.50	627.81
Female	Adult	1961	642.08	2243.97	2122.54	5595.77
Male	Adult	1961	272.89	787.06	3068.38	5311.42
Male	Adult	1961	27688.12	7432.26	500.52	7179.84
Unknown	Adult	1961	185780.36	16379.21	4910.70	5505.16
Male	Adult	1961	42873.22	41718.13	829.05	7880.66
Male	Adult	1961	31268.26	2990.04	671.66	5752.10
Male	Unknown	1962	7191.04	2468.44	240.39	6473.39
Unknown	Subadult	1962	9230.67	2961.75	1816.75	7230.73
Female	Adult	1962	10294.56	2386.83	107.96	5785.73
Male	Adult	1962	41.70	207.58	31.69	28.15
Male	Subadult	1963	337.42	607.06	122.11	6256.99
Male	Adult	1964	2269.83	2257.38	13.40	6827.23
Female	Subadult	1965	8069.74	2549.52	435.03	5468.22
Male	Unknown	1965	6289.84	1367.22	139.11	6880.36
Male	Unknown	1965	3021.88	2896.64	1201.88	6358.88
Male	Unknown	1965	5038.08	949.13	55.25	6060.81
Female	Unknown	1966	6243.97	3393.02	238.42	8522.71
Male	Subadult	1969	2066.88	1009.86	34.11	5522.29
Female	Adult	1970	3843.23	1661.89	77.21	6779.64
Female	Adult	1970	1635.92	670.46	407.64	5778.46
Female	Subadult	1971	30329.86	7621.84	10412.57	9460.71
Female	Adult	1972	743.85	1226.10	553.28	7455.46
Female	Subadult	1972	753.68	1231.11	152.38	6479.88
Female	Adult	1973	1727.08	808.36	43.57	6373.37

Appendix 3.2 continued:

WAL227.2	USNM500254	Smith: Mamm	St. Lawrence Island
WAL230.2	USNM500253	Smith: Mamm	Bering Sea
WAL078	12071	UAM: Mamm	Round Island
WAL089	12069	UAM: Mamm	Round Island
WAL093**	12070	UAM: Mamm	Round Island
WAL020.1**	12084	UAM: Mamm	Round Island/Nushagak Bay
WAL037	12074	UAM: Mamm	Round Island
WAL049.2	12082	UAM: Mamm	Round Island/Nushagak Bay
WAL058.2	12076	UAM: Mamm	Round Island/Nushagak Bay
WAL067	64189	UAM: Mamm	Savoonga
WAL070	12072	UAM: Mamm	Round Island
WAL071.2	12078	UAM: Mamm	Round Island/Nushagak Bay
WAL073	12083	UAM: Mamm	Round Island
WAL074	12073	UAM: Mamm	Round Island
WAL076**	12075	UAM: Mamm	Round Island
WAL079.1**	12079	UAM: Mamm	Round Island/Nushagak Bay
WAL080	12077	UAM: Mamm	Round Island
WAL082.2	12081	UAM: Mamm	Round Island/Nushagak Bay
WAL084.2	12080	UAM: Mamm	Round Island/Nushagak Bay
WAL087.2	12085	UAM: Mamm	Round Island/Nushagak Bay
WAL038.2	12086	UAM: Mamm	Round Island/Nushagak Bay
WAL046	14793	UAM: Mamm	Port Moller
WAL083**	24069	UAM: Mamm	Port Heiden

Male	Adult	1973	44.51	66.02	36.41	23.18
Male	Adult	1973	43.36	135.65	18.42	27.72
Male	Subadult	1977	253.13	280.48	36.63	5981.80
Male	Subadult	1977	98533.53	12644.41	4077.89	4299.91
Male	Subadult	1977	7577.75	2879.30	731.71	5844.89
Male	Subadult	1978	5086.36	2516.89	102.24	6385.93
Female	Adult	1978	121.86	895.42	86.20	6760.93
Male	Adult	1978	438.85	1043.57	107.19	6476.83
Male	Subadult	1978	429.81	1119.65	79.80	6679.27
Unknown	Subadult	1978	3069.66	3240.29	159.86	5878.87
Male	Subadult	1978	206.75	716.60	54.68	6666.06
Male	Adult	1978	351.36	573.07	42.85	5433.86
Male	Adult	1978	853.58	408.61	55.40	6033.95
Male	Subadult	1978	223.58	580.00	35.82	7395.81
Male	Subadult	1978	1147.65	533.79	81.07	6444.88
Male	Adult	1978	171.12	340.35	37.11	6385.59
Male	Adult	1978	836.94	140.19	25.12	6635.85
Male	Adult	1978	130.91	146.81	25.20	6240.82
Male	Unknown	1978	26990.14	2856.02	187.09	7642.11
Male	Adult	1978	53.59	105.48	16.19	7145.64
Male	Subadult	1979	166.24	604.63	35.97	6345.20
Male	Adult	1981	265.66	692.51	14.68	6213.04
Male	Adult	1981	837.30	352.23	64.91	6156.90

Appendix 3.3: **List of modern walrus samples with provenience and steroid hormone concentrations data.** Modern time period defined as bones collected in 2014 and 2015. Bones were collected in a collaborative effort between Alaskan subsistence hunters from Savoonga and Gambell, the Eskimo Walrus Commission, the U.S. Fish and Wildlife Service, and the Alaska Department of Fish and Game (ADF&G), Barrow subsistence hunters, and the North Slope Borough Department of Wildlife Management. **Indicates sample had duplicate bones analyzed for steroid hormone concentrations (progesterone (P), testosterone (T), cortisol (C), and estradiol (E)), and final concentrations were determined by averaging duplicate concentrations (ng/g lipid).

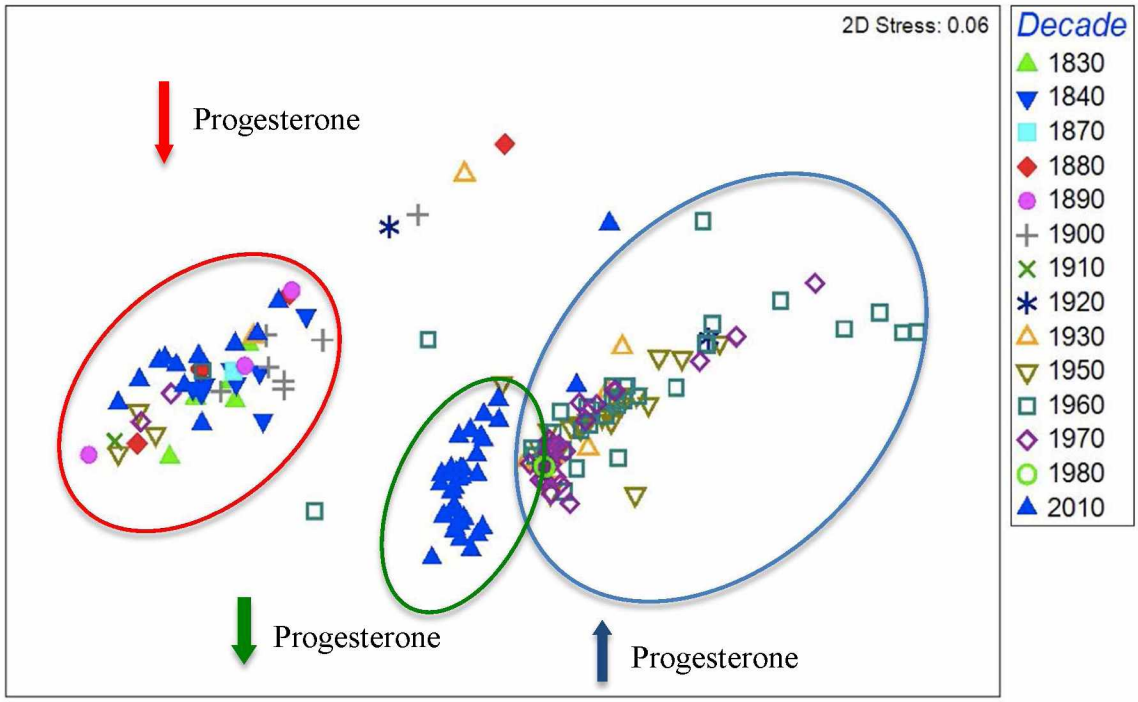
Modern Samples										
Project Number	**Catalog Number**	**Source**	**Location**	**Sex**	**Age Class**	**Year Collected**	**P [ng/g lipid]**	**T [ng/ g lipid]**	**C [ng/g lipid]**	**E [ng/g lipid]**
WAL125.1	S14-0001	Subsistence	Savoonga	Unknown	Adult	2014	430.60	334.66	153.35	2381.91
WAL126	S14-0002A	Subsistence	Savoonga	Male	Adult	2014	177.73	117.02	28.52	2204.44
WAL128.1	S14-0005	Subsistence	Savoonga	Male	Subadult	2014	1350.89	478.61	69.05	2507.32
WAL129.1	S14-0007	Subsistence	Savoonga	Unknown	Adult	2014	532.60	355.33	74.72	2421.35
WAL130.1	S14-0009	Subsistence	Savoonga	Male	Adult	2014	1867.40	544.63	51.73	2308.57
WAL131.1	S14-0010	Subsistence	Savoonga	Male	Adult	2014	750.91	1242.21	105.79	2179.38
WAL132.1	S14-0011	Subsistence	Savoonga	Female	Unknown	2014	440.70	1162.74	59.02	2080.20
WAL133.1	S14-0014	Subsistence	Savoonga	Male	Unknown	2014	225.23	255.88	169.63	2233.05
WAL134.1	S14-0017	Subsistence	Savoonga	Unknown	Adult	2014	323.67	364.51	636.55	2417.50
WAL135.1	S14-0018	Subsistence	Savoonga	Male	Adult	2014	583.23	315.14	669.50	2326.50
WAL136.1	S14-0019	Subsistence	Savoonga	Male	Adult	2014	1067.52	243.52	43.60	1984.32
WAL137.1	S14-0021	Subsistence	Savoonga	Male	Adult	2014	55.38	45.05	4.64	2400.36
WAL138.1	S14-0022	Subsistence	Savoonga	Male	Adult	2014	38.71	146.66	4.92	2505.16
WAL139.1	S14-0024	Subsistence	Savoonga	Male	Adult	2014	5464.69	1333.68	7.34	4030.24
WAL140.1	S14-0027	Subsistence	Savoonga	Female	Adult	2014	1526.27	722.84	63.37	2483.80
WAL141.1	S14-0029	Subsistence	Savoonga	Male	Adult	2014	395.55	302.16	165.50	2137.07
WAL142.1	S14-0034	Subsistence	Savoonga	Male	Adult	2014	511.31	334.89	135.30	2343.49
WAL143.1	S14-0035	Subsistence	Savoonga	Male	Adult	2014	625.95	377.46	457.87	2416.88
WAL144.1	S14-0036	Subsistence	Savoonga	Male	Adult	2014	2753.11	328.88	60.23	2634.24
WAL145	S14-0038	Subsistence	Savoonga	Male	Adult	2014	2572.37	279.55	38.92	2074.89
WAL146	S14-0039	Subsistence	Savoonga	Male	Adult	2014	1266.98	254.52	65.24	2637.05
WAL147.1	S14-0040	Subsistence	Savoonga	Male	Adult	2014	36.56	82.62	54.01	2373.95
WAL148	S14-0044	Subsistence	Savoonga	Male	Adult	2014	805.23	311.40	230.07	2343.89
WAL149.1	S14-0045	Subsistence	Savoonga	Female	Adult	2014	352.07	142.47	15.66	2370.49
WAL150	S14-0046	Subsistence	Savoonga	Male	Adult	2014	39.78	75.67	15.44	2363.43
WAL151.1	G14-0002	Subsistence	Gambell	Female	Adult	2014	283.70	234.97	349.71	2469.19
WAL152.1	G14-0005	Subsistence	Gambell	Male	Adult	2014	537.89	396.38	2458.07	2085.84
WAL153.1	G14-0011	Subsistence	Gambell	Female	Adult	2014	159.05	286.52	33.71	2567.74
WAL154.1	G14-0036	Subsistence	Gambell	Male	Adult	2014	230.43	100.17	15.87	2574.39
WAL155.1	G14-0042	Subsistence	Gambell	Female	Adult	2014	171.23	80.06	29.82	2483.24
WAL156.1	G14-0046	Subsistence	Gambell	Female	Adult	2014	209.46	139.07	15.08	2383.13
WAL157.1	G14-0048	Subsistence	Gambell	Female	Adult	2014	112.38	86.10	14.02	2364.61

Appendix 3.3 continued:

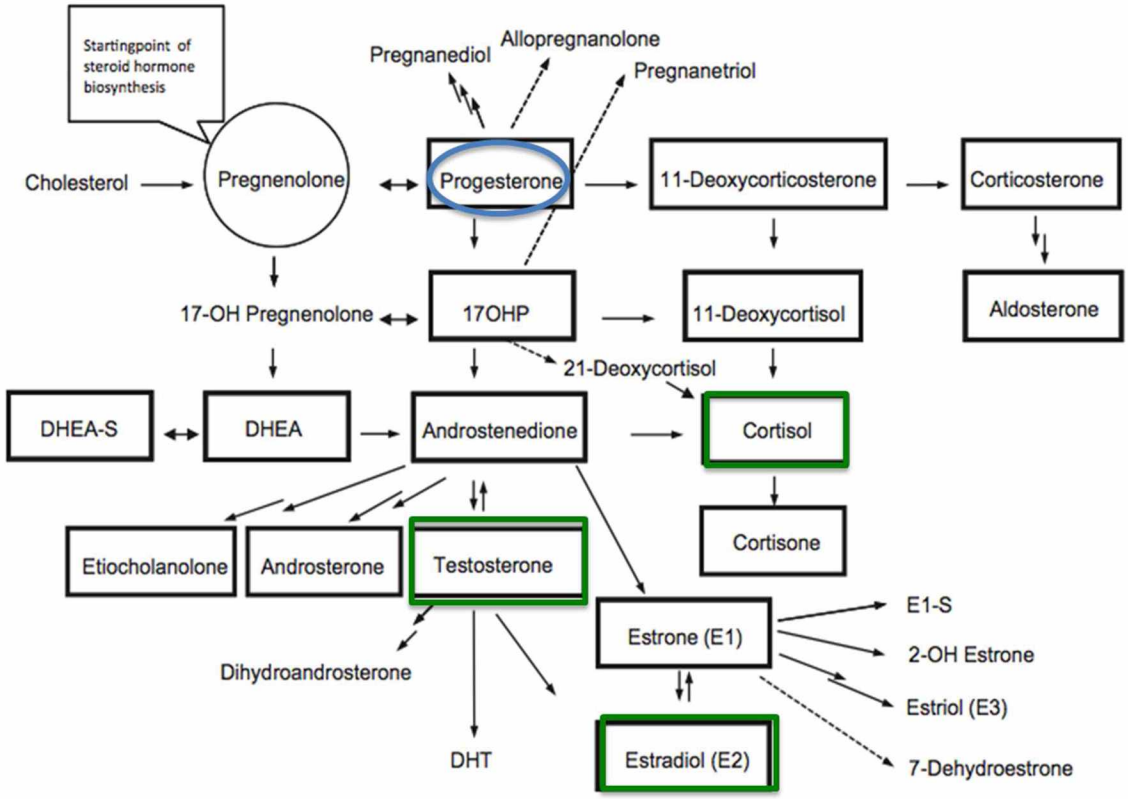
WAL189	S14-0002B	Subsistence	Savoonga	Male
WAL621	2014_W_23	Subsistence	Barrow	Male
WAL622	2014_W_24	Subsistence	Barrow	Female
WAL623	2014_W_25	Subsistence	Barrow	Female
WAL233	S15-027	Subsistence	Savoonga	Unknown
WAL234	S15-039	Subsistence	Savoonga	Male
WAL235**	S15-013	Subsistence	Savoonga	Male
WAL237	S15-036	Subsistence	Savoonga	Male
WAL238	S15-030	Subsistence	Savoonga	Male
WAL239	S15-009	Subsistence	Savoonga	Male
WAL240	S15-022	Subsistence	Savoonga	Male
WAL241	S15-037	Subsistence	Savoonga	Male
WAL282	G15-005	Subsistence	Gambell	Female
WAL283	G15-023	Subsistence	Gambell	Male
WAL284	G15-015	Subsistence	Gambell	Male

Adult	2014	2193.36	1188.16	202.28	2193.36
Adult	2014	18.71	98.03	27.63	2806.29
Adult	2014	76.30	14392.77	7395.37	2693.03
Adult	2014	13.47	76.68	20.21	1740.94
Adult	2015	24.52	392.97	281.40	29.76
Adult	2015	15.33	186.39	72.03	29.47
Adult	2015	35.77	253.51	141.11	34.83
Adult	2015	6.33	132.22	49.22	13.48
Adult	2015	3.49	135.44	59.16	17.54
Adult	2015	18.09	299.98	153.50	83.04
Adult	2015	3.72	100.02	43.97	20.47
Adult	2015	17.79	135.52	76.08	20.01
Adult	2015	127.73	108.69	58.98	29.79
Adult	2015	40.39	153.42	40.41	27.44
Adult	2015	11.98	68.77	36.35	20.16

Appendix 3.4: **nMDS plot of walrus samples grouped based on similar hormone concentrations and plotted by decade.** The blue ellipsis corresponds to decades (1950s – 1980s), where progesterone levels were highest (blue arrow). The green ellipsis represents the 2014 samples that had lower progesterone levels compared with the blue group (green arrow). The red ellipsis are the earliest samples from the 1880s – 1920s and the 2015 samples, that included mainly adult males (Appendices 3.2, 3.3) and had the lowest progesterone concentrations.



Appendix 3.5: **Steroid hormone pathways starting with cholesterol and leading to other steroid hormones.** Progesterone (blue circle) is the precursor to all other steroid hormones analyzed in this study (i.e., cortisol, testosterone, and estradiol; green highlight, after Koal et al. 2012).



Chapter 4: General Conclusions

Climate change in the Arctic is altering sea ice habitat of the Pacific walrus (*Odobenus rosmarus divergens*, hereafter walrus), and it is unclear how or if the walrus will be able to adapt to the rapidly changing Arctic climate. The loss of sea ice is altering benthic food web dynamics that could reduce walrus preferred benthic prey biomass (Grebmeier et al. 2006, Bluhm and Gradinger 2008, Grebmeier 2012). In addition, sea ice loss is forcing females and their calves to travel further distances to feeding areas in the Chukchi Sea (Metcalf and Robards 2008), increasing foraging distances to preferred summer feeding grounds (Jay et al. 2012), and decreasing calf survival (Cooper et al. 2006, Metcalf and Robards 2008). While many studies have focused on changes in walrus behavior in response to reduced sea ice in the Arctic (e.g., Cooper et al. 2006, Jay and Fischbach 2008, Jay et al. 2012), few studies have determined physiological effects of climate change on walruses. The overall objective of this thesis was to determine the stress response and reproductive status of the modern walrus population in response to climate change in the Arctic. A true physiological baseline, utilizing steroid hormones in archaeological walrus bone, was established and compared to bone steroid hormones measured in historic and modern walruses. This comparison allowed for the analyses of the physiological resilience (i.e., stress response and reproductive status) of walruses to climate change. In addition, bone steroid hormones were developed as a novel long-term physiological monitoring tool.

Serum and blubber are commonly used in steroid hormone studies to monitor the stress response and reproductive status of marine mammals (reviewed in Amaral 2010, Myers et al. 2010, Kellar et al. 2006, 2013, 2015, Zhang et al. 2014, Vu et al. 2015, Kershaw and Hall 2016), but this study is the first to use bone steroid hormones in marine mammal bone. Thus, it was

imperative to understand how walrus bone steroid hormone concentrations relate to commonly obtained tissues used in steroid hormone studies (i.e., blubber and serum). Bone steroid hormone concentrations are indicative of a long-term reservoir of steroid hormones; however, the exact reservoir time of specific steroid hormones is still unknown (Yarrow et al. 2010).

In chapter 2, we estimated that the complete cortical bone turnover in a walrus skeleton is approximately 33 years. Cortical bone's potential reservoir time of steroid hormones could be an accumulated average over the lifespan of a walrus (maximum age is approximately 40 years, Fay 1982). Our results showed that cortisol and estradiol measured in bone were similar to blubber hormone concentrations. Therefore, both bone and blubber are reliable long-term monitors of steroid hormones, and while blubber accumulates hormones over an estimated monthly time period (Kellar et al. 2006, 2009, 2013, 2015, Trana et al. 2015, Kershaw and Hall 2016), bone most likely accumulates hormones over a longer multiple year or decadal timescale. Progesterone in blubber of walrus females was significantly higher compared with males. This agrees with Kellar et al. (2006, 2013), and reflects the accumulated progesterone from the previous breeding season and pregnancies (Kellar et al. 2006, 2013). Progesterone in male walrus bone was elevated compared to females, possibly due to progesterone being an important precursor to testosterone (Wagner 2006). Overall, testosterone in bone of both sexes was higher compared with blubber and serum. Testosterone is a precursor to estradiol (Koal et al. 2012), and both hormones are important for bone remodeling and bone mineral density (Yarrow et al. 2010, Nguyen et al. 2014, During et al. 2015). Thus, the higher levels of testosterone in bone compared with blubber and serum could be a reservoir for maintaining bone health. Estradiol (but not the other measured steroid hormones) showed high interannual variability between 2014 and 2015 in all tissues. This result contrasts with our estimate of a lifetime accumulated average of estradiol

in walrus cortical bone. Estradiol is locally produced in bone (Yarrow et al. 2010), and as mentioned, has an important function in bone mineral density. Bone can therefore be considered an endocrine organ for estradiol, and estradiol having a relatively shorter reservoir time in bone compared with other hormones measured in this study. Overall, my results support bone as a long-term reservoir of steroid hormones compared with serum, which is a short-term pool of circulating hormones (Amaral 2010, Myers et al. 2010, Zhang et al. 2014).

Chapter 3 determined if bone steroid hormones could be a fundamental tool to understand the physiological resiliency of walruses to climate change in the Arctic. Reproductive (i.e., estradiol, progesterone, and testosterone) and stress (i.e., cortisol) hormones were extracted and measured in walrus bone from archaeological (3450 – 200 calendar years before present (BP)) and historical (200 – 20 BP) time periods, in addition to walrus bone from modern times (2014 – 2015). The stress response of walruses from modern time periods was similar to the stress response from the broad archaeological and historical time periods. When the historic time period was analyzed in greater detail, walruses from the 1950s and 1960s showed significantly higher bone cortisol concentrations compared with present-day walruses. Thus, the walrus population has experienced times of a substantially higher stress response in the past, but currently has a low and/or similar stress response to the walrus population from archaeological time periods. This indicates that walruses currently do not appear to exhibit a chronic stress response related to climate change or other stressors.

Detecting changes in reproductive hormone concentrations throughout archaeological, historical, and modern time periods combined with times of known population increases and decreases during the 1830s through 2010s helped establish the current reproductive status of modern-day walruses. Estradiol, progesterone, and testosterone concentrations were significantly

lower in walruses from the modern time period compared with reproductive hormone concentrations from the historical time period. Specifically, reproductive hormones were significantly higher in the 1950s through 1970s, which coincides with a rapid increase in the walrus population due to protection from commercial harvesting (Fay et al. 1989, 1997, Garlich-Miller et al. 2011). Further, progesterone, testosterone, and estradiol were significantly correlated with the minimum walrus population estimate. We can conclude that reproductive hormones from bone are reliable long-term monitors of the walrus population size and can give insight into walrus population dynamics.

Low reproductive hormone concentrations in modern-day walruses may either be indicative of low calf production and/or a population approaching or currently at carrying capacity. Reproductive hormone concentrations measured in archaeological bone were similar to modern walrus bone. While we do not have population estimates for walruses during prehistoric times, most likely they fluctuated around their carrying capacity of around 200000 - 250000 animals (Hufford and Loughlin 2009). It is plausible that the similarity among reproductive hormone concentrations from archaeological and modern times indicate that the current walrus population is at or near its carrying capacity. However, an accurate walrus population assessment is needed to confirm our interpretation of these data; a population assessment using genetic mark-recapture techniques is currently underway by the U.S. Fish and Wildlife Service. The increased cortisol concentrations in the 1950s and 1960s would most likely be due to the rapid rate of walrus reproduction, because cortisol concentrations increase in males and females during times of breeding (Bartsh et al. 1992, Hunt et al. 2014, Kershaw and Hall 2016). There could also be increased competition due to increasing walrus numbers, adding to the increased stress response during the 1950s – 1960s, but walrus populations were relatively low in prior decades

(Fay et al. 1989, 1997). This decrease in predation by walruses would have fostered a buildup in benthic biomass until the late 1970s and 1980s, when increasing numbers of walruses began to deplete clam populations (Lowry et al. 1980).

We would most likely document an increase stress response related to competition for resources in the 1980s and/or 1990s when measuring cortisol in walrus bone. However, we only had two samples from the 1980s, and no available samples from the 1990s. In contrast, current benthic resources, including bivalves, are at high abundance around critical walrus feeding areas in the Chukchi Sea (Schonberg et al. 2014). In addition, if walruses are at carrying capacity, based on the low reproductive hormones in the modern walrus bone, there would not be a rapid population increase, as observed in the 1950s – 1960s (Fay et al. 1989, 1997), thus explaining the lower stress response in modern walruses. While the current low stress response and low reproductive hormones of walruses support the idea of a population at carrying capacity, future physiological monitoring of the walrus population is warranted due to further reductions of sea ice extent and thickness in the Arctic and potentially increased use of terrestrial haul-outs (Jay et al. 2012, Overland et al. 2013).

There are aspects of this study that warrant further research, including bone physiology, bone endocrinology, reservoir / turnover times of different steroid hormones in cortical bone, and correlation of bone steroid hormones in walruses compared with sea ice extent. The rate at which steroid hormones are incorporated into bone is unknown (Yarrow et al. 2010). While this study estimated the walrus cortical bone turnover rate, a direct application to steroid hormone turnover rate may not be accurate for all steroid hormones. Specifically, estradiol is locally produced in bone (Yarrow et al. 2010, this study), and this could lead to complications when determining the reservoir time and/or turnover of estradiol in cortical bone. Experimental studies to establish

steroid hormone turnover rates in bone could be done with laboratory mammals (e.g., mice, rats). Isotopically labeled steroid hormones could be injected into the bloodstream of laboratory mammals, and the amount of isotopically labeled hormone that is incorporated in and/or mobilized from cortical bone can be measured over multiple years. Thus, a more accurate steroid hormone turnover rate in cortical bone (compared with only cortical bone turnover rate) can be determined. However, realistically, these types of studies will not be feasible in marine mammals, and there are a variety of unique aspects of walrus bone that could make these studies on laboratory mammals not directly transferrable to marine mammals.

Compared to laboratory rats, shallow marine mammal divers (e.g., walrus) have high density bones, which aid as a ballast to maintain neutral buoyancy in water (Pond 1978, Gray et al. 2007), exhibit a lower mass-specific metabolic rate (Savage et al. 2007), and maintain a larger fat reservoir in bone (Pond 1978). While direct application of steroid hormone turnover rates / reservoir times in laboratory mammals may not be ideal for walruses, it would greatly enhance the general knowledge of physiology of steroid hormones in cortical bone.

It is well known that the life history of the walrus is tied to formation and melting of sea ice (Fay 1982, Metcalf and Robards 2008, Jay et al. 2012, Schonberg et al. 2014). Increased sampling during archaeological and historical time periods of known sea ice extent prior to current rapid climate change in the Arctic would be beneficial in determining if and/or how changes in sea ice extent relate to walrus physiology. Modern satellite documentation of sea ice extent goes back to 1979 (Mahoney et al. 2011), however substantial warming of the Arctic has occurred since the 1950s (Kaufman et al. 2009). Thus, there is not an accurate baseline of sea ice extent prior to the current warming in the Arctic. However, proxies using whaling logbooks can give estimates of sea ice extent from hundreds of years ago (Mahoney et al. 2011) and sediment

cores can give estimates of sea ice extent going back thousands of years (De Vernal et al. 2005, Kinnard et al. 2011).

If more archaeological walrus bone samples can be obtained before, during, and after the natural warming and cooling events in the Arctic (De Vernal et al. 2005), a correlation among steroid hormones measured in walrus bone and sea ice extent can be established. Additional samples have since been discovered, and are in the process of being sampled and analyzed to look at the correlation of bone steroid hormones and sea ice extent for walruses. This correlation could give an accurate picture of the relationship between walrus physiological parameters and sea ice extent. However, males do not routinely migrate to the Arctic in the summer as the walrus females and calves do, but instead migrate to Bristol Bay and the Gulf of Anadyr in the Northern Bering Sea (Fay 1982). Testing the correlation of steroid hormones measured in archaeological female walrus bone with sea ice extent would be the most beneficial. Bone steroid hormone investigations, similar to this study, can be applied to other marine mammals that are forecasted to be affected by climate change in the Arctic, most notably the polar bear (*Ursus maritimus*), bearded seal (*Erignathus barbatus*), ringed seal (*Pusa hispida*), and other ice seal species.

For future monitoring of stress and reproductive hormones in walruses, blubber would be a more plausible tissue to obtain compared with bone, where a hunter bias is present and the walrus has to be deceased. The application of walrus blubber to monitor steroid hormones is promising, and would enable minimally invasive biopsy sampling of free-ranging walruses. However, there are many aspects of marine mammal blubber that need to be considered before its use in routine steroid hormone monitoring. Blubber is a highly developed tissue that plays a critical role in thermoregulation, storage of lipids for energy, buoyancy, and contributes

structural support for a diving marine mammal (Biuw et al. 2003, Strandberg et al. 2008). These different functions of blubber result in different fatty acid compositions throughout the vertical thickness of blubber (Strandberg et al. 2008). Biopsy darts generally do not obtain a full thickness blubber core and increased soaking time in the ocean (due to difficulties in biopsy retrieval) could significantly lower hormone concentrations (Allen et al. 2015).

In chapter 2, we determined a significant correlation among progesterone measured in blubber and bone. However, the correlation was between progesterone concentrations measured in full thickness blubber and cortical bone. Thus, research is needed to determine if steroid hormones are similar at different body sites and differing blubber depths of walruses. In ringed seals, concentrations of specific fatty acids were higher in fat deposits located in the head and whisker region compared with the midbody fatty acid concentrations (Käkelä and Hyvärinen 1996). However, this is not always the case, as blubber fatty acid and contaminant profiles were similar among body sites in various cetaceans (Gauthier et al. 1997, Samuel and Worthy 2004). Similar studies measuring hormone concentrations in blubber at different sites on a walrus would be beneficial when comparing hormone concentrations from blubber biopsy samples taken from various body locations.

Additional information must also be collected to determine if steroid hormone concentrations in the top layer of walrus blubber (portion of the blubber layer most likely obtained by biopsy) are similar to steroid hormones measured in the lower portion of the blubber layer. Generally, fatty acids deposited close to muscle play a role in energy mobilization, and fatty acids located closer to the skin assist in thermoregulation (Koopman et al. 1996, Strandberg et al. 2008). In walruses, % lipid was not significantly different between the outer and inner portions of full thickness blubber (Seymour 2014). In addition, testosterone concentrations

measured in short beaked dolphins (*Delphinus delphis*) were similar among the upper, middle, and lower portions of the blubber layer (Kellar et al. 2009). Seymour (2014) and Kellar et al. (2009) results lend support to the notion that steroid hormones are being distributed and turned over similarly throughout the walrus blubber, although walrus-specific research is needed relating to steroid hormone concentrations among vertical blubber thickness.

This study has established baselines for stress and reproductive hormones of the Pacific walrus population based on hormones extracted from walrus bone during archaeological time periods, when the currently observed rapid climate change in the Arctic has not been documented. In addition, this study established the current stress response and reproductive status of the walrus population that can serve as a reference to future monitoring efforts. Continued monitoring is warranted considering slow steroid hormone turnover in bone, current low reproductive hormone concentrations, increasing land use by walruses and new haul-out locations (Jay et al. 2012, Kryukova et al. 2014), and the potential of a completely ice-free Arctic in 25 years (Overland et al. 2013). Walruses are experiencing an uncertain future due to vanishing sea ice habitat. However, this study supports the idea of walruses as a resilient Arctic marine mammal with many challenges ahead. However, with consistent future physiological monitoring, we can understand if and how this iconic Arctic marine mammal adapts to life without sea ice.

4.1 References

Allen, C., N. Beaulieu, M. Robbins, K. Catelani, M. L. Trego, and N. M. Kellar. 2015. Effects of blubber collection procedure and seawater exposure in relation to lipid loss and hormone levels. Oral presentation at the 21st Biennial Conference on the Biology of Marine Mammals, San Francisco, CA. December 17, 2015.

Amaral, R. 2010. Use of alternative matrices to monitor steroid hormones in aquatic mammals: A review. Aquatic Mammals 36:162–171.

Bartsh, S. S., S. D. Johnston, and D. B. Siniff. 1992. Territorial behavior and breeding frequency of male Weddell seals (*Leptonychotes weddelli*) in relation to age, size, and concentrations of serum testosterone and cortisol. Canadian Journal of Zoology 70:680–692.

Biuw, M., B. McConnell, C. J. Bradshaw, H. Burton, and M. Fedak. 2003. Blubber and buoyancy: monitoring the body condition of free-ranging seals using simple dive characteristics. Journal of Experimental Biology 206:3405–3423.

Bluhm, B., and R. Gradinger. 2008. Regional variability in food availability for Arctic marine mammals. Ecological Applications 18:77–96.

Cooper, L. W., C. J. Ashjian, S. L. Smith, L. A. Codispoti, J. M. Grebmeier, R. G. Campbell, and E. B. Sherr. 2006. Rapid seasonal sea-ice retreat in the Arctic could be affecting Pacific walrus (*Odobenus rosmarus divergens*) recruitment. Aquatic Mammals 32:98–102.

De Vernal, A., C. Hillaire-Marcel, and D. A. Darby. 2005. Variability of sea ice cover in the Chukchi Sea (western Arctic Ocean) during the Holocene. Paleoceanography doi:10.1029/2005PA001157

During, A., G. Penel, and P. Hardouin. 2015. Understanding the local actions of lipids in bone physiology. Progress in Lipid Research 59:126–146.

Fay, F. H. 1982. Ecology and biology of the Pacific walrus, *Odobenus rosmarus divergens* Illiger. North American Fauna 74:1–266.

Fay, F. H., B. P. Kelly, and J. L. Sease. 1989. Managing the exploitation of Pacific walruses: a tragedy of delayed response and poor communication. Marine Mammal Science 5:1–16.

Fay, F. H., L. L. Eberhardt, B. P. Kelly, J. J. Burns, and L. T. Quakenbush. 1997. Status of the Pacific walrus population, 1950-1989. Marine Mammal Science 13:537–565.

Garlich-Miller, J. L., J. G. MacCracken, J. Snyder, R. Meehan, M. Myers, J. M. Wilder, E. Lance, and A. Matz. 2011. Status review of the Pacific walrus (*Odobenus rosmarus divergens*). U.S. Fish and Wildlife Service, Marine Mammals Management, Anchorage, AK.

Gauthier, J. M., C. D. Metcalfe, and R. Sears. 1997. Validation of the blubber biopsy technique for monitoring of organochlorine contaminants in balaenopterid whales. Marine Environmental Research 43:157–179.

Gray, N. M., K. Kainec, S. Madar, L. Tomko, and S. Wolfe. 2007. Sink or swim? Bone density as a mechanism for buoyancy control in early cetaceans. Anatomical Record 290:638–653.

Grebmeier, J. M., L. W. Cooper, H. M. Feder, and B. I. Sirenko. 2006. Ecosystem dynamics of the Pacific-influenced northern Bering and Chukchi Seas in the Amerasian Arctic. Progress in Oceanography 71:331–361.

Grebmeier, J. M. 2012. Shifting patterns of life in the pacific Arctic and sub-Arctic Seas. Annual Review of Marine Science 4:63–78.

Hufford, G., and T. Loughlin. 2009. Bering Sea: marine mammals in a regional sea. Pages 172–204 *in* G. Carleston Ray and J. McCormick-Ray, editors. Coastal-Marine Conservation: Science and Policy. Blackwell Science Ltd, Victoria, Australia.

Hunt, K. E., R. Stimmelmayr, C. George, C. Hanns, R. Suydam, H. Brower Jr, and R. M. Rolland. 2014. Baleen hormones: a novel tool for retrospective assessment of stress and reproduction in bowhead whales (*Balaena mysticetus*). Conservation Physiology 2:1–12.

Jay, C. V., and Fischbach A. S. 2008. Pacific walrus response to Arctic Sea ice losses. U.S. Geological Survey, Fact Sheet 2008–3041. 4 pp.

Jay, C. V., A. S. Fischbach, and A. A. Kochnev. 2012. Walrus areas of use in the Chukchi Sea during sparse sea ice cover. Marine Ecology Progress Series 468:1–13.

Käkelä, R., and H. Hyvärinen. 1996. Site-specific fatty acid composition in adipose tissues of several northern aquatic and terrestrial mammals. Comparative Biochemistry and Physiology 115:501–514.

Kaufman, D. S., D. P. Schneider, N. P. Mckay, C. M. Ammann, R. S. Bradley, K. R. Briffa, G. H. Miller, B. L. Otto-Bliesner, J. T. Overpeck, B. M. Vinther, A. Lakes, and P. Members. 2009. Recent warming reverses long-term Arctic cooling. Science 325:4–7.

Kellar, N. M., M. L. Trego, C. I. Marks, and A. E. Dizon. 2006. Determining pregnancy from blubber in three species of delphinids. Marine Mammal Science 22:1–16.

Kellar, N. M., M. L. Trego, C. I. Marks, S. J. Chivers, K. Danil, and F. I. Archer. 2009. Blubber testosterone: A potential marker of male reproductive status in short-beaked common dolphins. Marine Mammal Science 25:507–522.

Kellar, N. M., J. Keliher, M. L. Trego, K. N. Catelani, C. Hanns, J. C. George, and C. Rosa. 2013. Variation of bowhead whale progesterone concentrations across demographic groups and sample matrices. Endangered Species Research 22:61–72.

Kellar, N. M., K. N. Catelani, M. N. Robbins, M. L. Trego, C. D. Allen, K. Danil, and S. J. Chivers. 2015. Blubber cortisol: A potential tool for assessing stress response in free-ranging dolphins without effects due to sampling. PLoS ONE 10:1–16.

Kershaw, J. L., and A. J. Hall. 2016. Seasonal variation in harbour seal (*Phoca vitulina*) blubber cortisol - A novel indicator of physiological state? Scientific Reports 6:21889.

Kinnard, C., C. M. Zdanowicz, D. A. Fisher, E. Isaksson, A. De Vernal, and L. G. Thompson. 2011. Reconstructed changes in Arctic sea ice over the past 1,450 years. Nature 479:509–512.

Koal, T., D. Schmiederer, H. Pham-Tuan, C. Röhring, and M. Rauh. 2012. Standardized LC-MS/MS based steroid hormone profile-analysis. Journal of Steroid Biochemistry and Molecular Biology 129:129–138.

Koopman, H. N., S. J. Iverson, and D. E. Gaskin. 1996. Stratification and age-related differences in blubber fatty acids of the male harbour porpoise (*Phocoena phocoena*). Journal of Comparative Physiology B 165:628–639.

Kryukova, N. V, A. A. Kochnev, and A. A. Pereverzev. 2014. The influence of ice conditions on terrestrial haulouts of the pacific walrus *Odobenus rosmarus divergens* Illiger, 1815 in the Gulf of Anadyr, Bering Sea. Russian Journal of Marine Biology 40:30–35.

Lowry, L. F., K. J. Frost, and J. J. Burns. 1980. Feeding of bearded seals in the Bering and Chukchi Seas and trophic interaction with Pacific walruses. Arctic 33:330–342.

Mahoney, A. R., J. R. Bockstoce, D. B. Botkin, H. Eicken, and R. A. Nisbet. 2011. Sea-ice distribution in the Bering and Chukchi seas: Information from historical Whaleships' logbooks and journals. Arctic 64:465–477.

Metcalf, V., and M. Robards. 2008. Sustaining a healthy human-walrus relationship in a dynamic environment: challenges for comanagement. Ecological Applications 18:148–156.

Myers, M. J., B. Litz, and S. Atkinson. 2010. The effects of age, sex, season and geographic region on circulating serum cortisol concentrations in threatened and endangered Steller sea lions (*Eumetopias jubatus*). General and Comparative Endocrinology 165:72–77.

Nguyen, H. T. T., B. Von Schoultz, T. V Nguyen, T. X. Thang, T. T. Chau, P. T. M. Duc, and A. L. Hirschberg. 2014. Sex hormone levels as determinants of bone mineral density and osteoporosis in Vietnamese women and men. Journal of Bone and Mineral Metabolism 33:658–665.

Overland, J. E., M. Wang, J. E. Walsh, and J. C. Stroeve. 2013. Future Arctic climate changes : Adaptation and mitigation time scales. Earth's Future 2:68–74.

Pond, C. M. 1978. Morphological aspects and the ecological and mechanical consequences of fat deposition in wild vertebrates. Annual Review of Ecology, Evolution, and Systematics 9:519–570.

Samuel, A. M., and G. A. Worthy. 2004. Variability in fatty acid composition of bottlenose dolphin (*Tursiops truncatus*) blubber as a function of body site, season, and reproductive state. Canadian Journal of Zoology 82:1933–1942.

Savage, V. M., A. P. Allen, J. H. Brown, J. F. Gillooly, A. B. Herman, W. H. Woodruff, and G. B. West. 2007. Scaling of number, size, and metabolic rate of cells with body size in mammals. Proceedings of the National Academy of Sciences of the United States of America 104:4718–4723.

Schonberg, S. V., J. T. Clarke, and K. H. Dunton. 2014. Distribution, abundance, biomass and diversity of benthic infauna in the Northeast Chukchi Sea, Alaska: Relation to environmental variables and marine mammals. Deep-Sea Research Part II: Topical Studies in Oceanography 102:144–163.

Seymour, J. 2014. Pacific walrus use of higher trophic level prey and the relation to sea ice extent, body condition, and trichinellosis. Dissertation. University of Alaska Fairbanks, Fairbanks, AK.

Strandberg, U., A. Käkelä, C. Lydersen, K. M. Kovacs, O. Grahl-Nielsen, H. Hyvärinen, and R. Käkelä. 2008. Stratification, composition, and function of marine mammal blubber: the ecology of fatty acids in marine mammals. Physiological and Biochemical Zoology 81:473–485.

Trana, M. R., J. D. Roth, G. T. Tomy, W. G. Anderson, and S. H. Ferguson. 2015. Increased blubber cortisol in ice-entrapped beluga whales (*Delphinapterus leucas*). Journal of Experimental Marine Biology and Ecology 462:8–13.

Vu, E. T., C. Clark, K. Catelani, N. M. Kellar, and J. Calambokidis. 2015. Seasonal blubber testosterone concentrations of male humpback whales (*Megaptera novaeangliae*). Marine Mammal Science 31:1258–1264.

Wagner, C. K. 2006. The many faces of progesterone: A role in adult and developing male brain. Frontiers in Neuroendocrinology 27:340–359.

Yarrow, J. F., C. F. Conover, J. A. Lipinska, C. A. Santillana, T. J. Wronski, and S. E. Borst. 2010. Methods to quantify sex steroid hormones in bone: applications to the study of androgen ablation and administration. American Journal of Physiology - Endocrinology and Metabolism 299:E841–E847.

Zhang, P., Y. Yang, J. Han, Z. Lu, L. Wang, J. Tian, and Q. Wang. 2014. Serum testosterone, progesterone, and estradiol concentrations and sexual maturation in spotted seals (*Phoca largha*). Theriogenology 82:475–480.e4.